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ABSTRACT

This manual presents laboratory procedures for the differentiation and identification of disease agents from clinical materials. Included are procedures for the collection of specimens, preparation of culture media, pure culture methods, cultivation of the microorganisms in natural and simulated natural environments, and procedures in identification. Also included are sensitivity determinations, safety practices in the clinical laboratory, and clinical laboratory monitoring of nosocomial diseases. For the more frequently performed tests, the principle of the test, the reagents and equipment needed, the preliminary steps, complete procedures, and interpretation of results are included. (Author/CS)

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CLINICAL LABORATORY PROCEDURES: BACTERIOLOGY

3 October 1977

DEPARTMENTS OF THE AIR FORCE AND THE ARMY

DEPARTMENTS OF THE AIR FORCE
AND THE ARMY
Washington DC 20330

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FM 8–65
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Medical Service

CLINICAL LABORATORY PROCEDURES: BACTERIOLOGY

This manual presents laboratory procedures for the differentiation and identification of disease agents from clinical materials. Included are procedures for the collection of specimens, preparation of culture media, pure culture methods, cultivation of the microorganisms in natural and simulated natural environments, and procedures in identification. Also included are sensitivity determinations, safety practices in the clinical laboratory, and clinical laboratory monitoring of nosocomial diseases. For the more frequently performed tests, the manual presents the principle of the test, the reagents and equipment needed, the preliminary steps, the complete procedure, and interpretation of results.

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Chapter 1

INTRODUCTION TO BACTERIOLOGY

1–1. Introduction. Bacteriology is the science that deals with the microscopic, single-celled bodies called bacteria. Some of these microorganisms are useful in agriculture and industry, while others are capable of causing infection or disease in the human body. It is with the latter, the pathogens, that this manual is concerned. Since accurate clinical diagnosis of bacterial disease is difficult, laboratory confirmation of the clinical diagnosis is very important. The primary purpose of this manual is to serve as a ready reference for the laboratory technician, whose task it is to obtain a clinical specimen, prepare a pure culture of the organism from the clinical specimen, and examine the culture for the purpose of identification.

1–2. Taxonomy of Bacteria:

a. The orderly classification of plants and animals according to their presumed natural relationships is known as the science of taxonomy. The basic concept of taxonomy is that all living things have a definite and logical place in the universal scheme of things. Originally, all living organisms were placed in one of two kingdoms: the Plant Kingdom or the Animal Kingdom. From the beginning, the classification of bacteria presented problems because bacteria have similarities to both kingdoms. For example, the Actinomyces resemble fungi in many respects; other bacteria, the Mycobacteria (tubercle-related bacilli) and Corynebacteria (diphtheria and diphtheroid bacilli, for example) resemble members of the Plant Kingdom. Still other bacteria, the protozoa and to a lesser extent the spirochetes, resemble members of the Animal Kingdom. Early classification placed bacteria in the Plant Kingdom, but recently biologists have agreed on a new classification which divides all cellular life (as opposed to virus life) into two groupings on the basis of cellular organization. One grouping includes those cells that have a nucleus separated from the cytoplasm by a membrane, and the other is made up of those that have cells without a nucleus. The new *Bergey's Manual of Determinative Bacteriology** refers to this third kingdom, composed of organisms having cells without nuclei, as the Kingdom Procaryotae. Those organisms having cells with a nucleus are referred to as Eucaryotae. The Plant and Animal Kingdoms have not been combined as yet into a single kingdom and given new nomenclature because of the difficulties involved in rearranging the hierarchies. There are other distinctions between procaryotes and eucaryotes, but cellular organization is the essential feature upon which the classification is made.

b. The Kingdom Procaryotae is further divided into divisions I and II, as follows:

(1) Division I, Phototrophic Procaryotes, the blue-green algae, which are capable of aerobic photosynthesis.

(2) Division II, Procaryotes indifferent to light, referred to as "Scotobacteria", which includes most of the bacteria encountered in the clinical laboratory.

c. Division II is subdivided into three classes:

(1) Class I. The bacteria—present Parts 2 to 17.

(2) Obligate intracellular Scotobacteria in eucaryotic cells—Rickettsias—present Part 18.

(3) Scotobacteria without cell walls—Mollicutes—present Part 19.

d. Each Part is further divided into orders, families, and genera. Figure 1–1 illustrates the new schema. Table 1–1 compares the classification of a bacterium under the old and new taxonomic arrangements.

1–3. Classification by Characteristics. The clinical laboratory technician uses a combination of observations and tests to place a specimen

Table 1–1. Comparison of Classification of Bacterium Under Old and New Taxonomy.

PLANT KINGDOM (OLD)		
	Division I	Protophyta (primitive plants, bacteria)
	Division II	Thallophyta (algae, fungi, lichens)
	Division III	Bryophyta (liverworts, mosses)
	Division IV	Pteridophyta (ferns, club mosses)
	Division V	Spermatophyta (seed plants, trees)
KINGDOM PROCARYOTAE (NEW)		
	Division I	The Cyanobacteria
	Division II	The Bacteria (organisms placed according to similarities into Parts, Order, Family and Genus)

*Bergey's Manual of Determinative Bacteriology, Eighth Edition. Copyright 1974 by The Williams and Wilkins Company.

KINGDOM PROCARYOTAE

Division I. The Cyanobacteria
Division II. The Bacteria

PART 1. PHOTOTROPIC BACTERIA

PART 2. THE GLIDING BACTERIA

PART 3. THE SHEATHED BACTERIA

PART 4. BUDDING and/or APPENDAGED BACTERIA

PART 5. THE SPIROCHETES

PART 6. SPIRAL AND CURVED BACTERIA

PART 7. GRAM-NEGATIVE ANAEROBIC RODS AND COCCI
Family I. *Pseudomonadaceae*
Genus I. *Pseudomonas*
Genera of Uncertain Affiliation
Genus *Alcaligenes*
Genus *Achromobacter*
Genus *Brucella*
Genus *Bordetella*
Genus *Francisella*

PART 8. GRAM-NEGATIVE FACULTATIVELY ANAEROBIC RODS
Family I. *Enterobacteriaceae*
Genus I. *Escherichia*
Genus II. *Edwardsiella*
Genus III. *Citrobacter*
Genus IV. *Salmonella*
Genus V. *Shigella*
Genus VI. *Klebsiella*
Genus VII. *Enterobacter*
Genus VIII. *Hafnia*
Genus IX. *Serratia*
Genus X. *Proteus*
Genus XI. *Yersinia*
Genus XII. *Erwinia*
Family II. *Vibrionaceae*
Genus I. *Vibrio*
Genera of Uncertain Affiliation
Genus *Haemophilus*
Genus *Pasteurella*

PART 9. GRAM-NEGATIVE ANAEROBIC BACTERIA
Family I. *Bacteroidaceae*
Genus I. *Bacteroides*

PART 10. GRAM-NEGATIVE COCCI AND COCCOBACILLI
Family I. *Neisseraceae*
Genus I. *Neisseria*
Genus II. *Branhamella*
Genus III. *Moraxella*
Genus IV. *Acinetobacter*

PART 11. GRAM-NEGATIVE ANAEROBIC COCCI
Family I. *Veillonellaceae*
Genus I. *Veillonella*

PART 12. GRAM-NEGATIVE, CHEMOLITHOTROPHIC BACTERIA

PART 13. METHANE-PRODUCING BACTERIA

PART 14. GRAM-POSITIVE COCCI
a. Aerobic and/or facultatively anaerobic
Family I. *Micrococcaceae*
Genus I. *Micrococcus*
Genus II. *Staphylococcus*
Family II. *Streptococcaceae*
Genus I. *Streptococcus*
Genus V. *Gemella*
b. Anaerobic
Family III. *Peptococcaceae*
Genus I. *Peptococcus*
Genus II. *Peptostreptococcus*
Genus IV. *Sarcina*

PART 15. ENDOSPORE-FORMING RODS AND COCCI
Family I. *Bacillaceae*
Genus I. *Bacillus*
Genus III. *Clostridium*

PART 16. GRAM-POSITIVE ASPOROGENOUS ROD-SHAPED BACTERIA
Genera of Uncertain Affiliation
Genus *Listeria*
Genus *Erysipelothrix*

PART 17. ACTINOMYCETES AND RELATED ORGANISMS
Coryneform Group of Bacteria
Genus I. *Corynebacterium*
Order I. *Actinomycetales*
Family I. *Actinomycetaceae*
Genus I. *Actinomyces*
Genus III. *Bifidobacterium*
Family II. *Mycobacteriaceae*
Genus I. *Mycobacterium*
Family VI. *Nocardiaceae*
Genus I. *Nocardia*

PART 18. THE RICKETTSIAS

PART 19. THE MYCOPLASMAS

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Figure 1–1. Illustration of New Schema.

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in a certain location or category in the taxonomic system and eventually identify it. The first factor that aids in the identification of a strain is information as to where it was obtained and under what conditions. Next, careful morphological examination of the specimen under the microscope with observations of size, shape, cell arrangement, and cell structures together with details as to colonial morphology and gram-staining reactions, narrows the search to a small group of genera. Finally, biochemical tests, for example, tests for nutritional requirements, sugar utilization, and metabolic products, complete the identification.

1–4. Microscopic Morphology. With the aid of the microscope, the bacteriologist can view bacteria and determine their *size*, *shape*, and

cell structure; then separate them into specific groups or genera, using any similarities as his criterion. Cell structures have become more prominent and useful in classification with the higher magnifications possible since the advent of the electron microscope. Morphological aspects cannot be used as the sole basis for classification of bacteria because organisms may be morphologically alike but quite different physiologically. However, morphology cannot be discounted as an integral part of the classification process. The aspects of morphology that are of significance in classifying and identifying an organism will be considered now in more detail.

a. **Size.** The unit of measurement used in measuring bacteria is the micrometer (formerly called the micron). A micrometer (micron) is equal to one-one-millionth of a meter (1 μm = 0.000001 M). Generally, bacteria range in size from about 0.5 μm to 2.0 μm in diameter and from 7 μm to 8 μm in length depending on the genus or species. There is, however, no definite size applicable to a specific genus. For example, the diameter of *Staphylococcus aureus* may be from 0.3 μm to 1.0 μm. Even within a species, variations in size are not uncommon. *Bacillus subtillis* may be from 0.7 μm to 0.8 μm in diameter and from 2 μm to 3 μm in length. *Bacillus megaterium* ranges from 1.2 μm to 1.5 μm in diameter and from 2 μm to 5 μm in length.

b. **Shapes.** The shape of a particular bacterium is frequently characteristic of the genus. For example, members of the genus *Clostridium* are rod-shaped with spores that are generally wider than the diameter of the rods in which they develop. On the other hand, members of a single family or genus may vary as to shape or cellular arrangement. Bacteria may be spherical, rodlike, or spiral in shape, and the cells may occur singly or may be arranged in pairs, chains, clusters, tetrads, or cubes (see figure 1–2).

(1) *Spherical Shapes or Cocci.* Cocci may be separated according to their arrangement as diplococci, streptococci, or staphylococci. Cellular arrangement is the result of the type of cellular division. Both the diplococci and the streptococci are the result of cellular division along one plane, but the diplococci remain attached in pairs following cell division (for example, *Streptococcus faecalis*). There are other arrangements besides those already named, such as the *Gaffkya* sp and the genus *Sarcina*. In the *Gaffkya*, cell division occurs in two planes, and cells remain attached in tetrads. In the *Sarcina*, division of cells takes place in three planes, and cells remain attached in packets of eight.

(2) *Rodlike Shapes or Bacilli.* The bacilli vary in shape or form. In addition to the typical rodlike shape, some species have a slightly curved or ellipsoid form, intermediate between the cocci and the bacilli. This shape is referred to as coccobacillus (figure 1–2). An example is the saprophytic species of the genus *Corynebacterium*, called diphtheroids. Other bacilli produce atypical or unusual forms, such as fusiforms or cigar-shaped forms tapering toward each end, comma shapes, club shapes, and V- or Y-shaped branching forms. These unusual forms are demonstrated by the nonacid-fast organism of the Actinomyces, which first produces a vegetative mycelium and upon aging, breaks up into bacillary or coccoid elements. The bacilli divide in one plane vertical to the long axis. They may be classified on the basis of cellular arrangement as bacilli, diplobacilli, streptobacilli, or palisades. The most common arrangement is the single cell arrangement that occurs in the bacilli. In the diplobacilli, the cells occur in pairs, and in the streptobacilli, they remain attached after division in chains, ranging from short chains to extremely long ones. The palisade arrangement is one in which several cells line up side by side due to postfission slipping. This arrangement is found in the diphtheroids.

(3) *Spiral Shapes or Spirilla.* Spirilla (singular form: spirillum) are spiral or helical in shape. They are actually rodlike forms which undergo coiling. Spirilla are the typical form of members of the families Spirillaceae and Spirochaetaceae. Organisms of these families reproduce by transverse fission. In the *Spirillaceae*, short chains are sometimes formed, while in the Spirochaetaceae, only single cells are seen (see figure 1–2).

c. **Cell Structures:**

(1) *Basic Structures.* As discussed previously (paragraph 1–2 of this chapter), the chief distinction between the Procaryotae and Eucaryotae is the presence or absence of a well-defined nucleus enclosed in a cytoplasmic membrane. Instead of a nucleus, the procaryotes possess a genophore consisting of a two-stranded DNA (deoxyribonucleic acid) fibril. The cytoplasm that surrounds the genophore is not bounded by a cytoplasmic membrane. The lone membrane existing in the procaryotic cell is the plasma membrane enclosing the protoplasm. Even the cell wall is not present in some organisms, such as mem-

Figure 1-2. Bacterial Shapes.

bers of the genera *Mycoplasm* and *Halobacterium*.

(2) *Other Structures.* Specialized structures, such as the capsule, flagellum, spore, and inclusion body, may be found in the cells of certain bacteria. These features can be used in differentiation and identification of various organisms.

(a) *Capsule.* The capsule is a structure that can be found surrounding a single bacterium or a clump of bacteria. It is composed of a polysaccharide substance high in molecular weight. The structure serves as a defense against phagocytosis (engulfment by white cells). Usually the presence of the capsule is an indication of virulence.

(b) *Flagellum.* The flagellum is a protoplasmic strand of elastic protein originating in the cytoplasm and extending from the body of the cell. The undulating, wavelike motion of the flagella affords the chief means of locomotion of the motile bacteria. The number and arrangement of flagella help in the classification of strains. Only rodlike bacteria (bacilli)

and spiral bacteria (spirochetes) possess flagella. Special terms have been developed to indicate the arrangement and location of flagella (see figure 1–3). The various arrangements of flagella are as follows:

1. *Monotrichous.* A single flagellum attached to one pole of a cell.

2. *Amphitrichous.* One to several flagella attached to each end of the cell.

3. *Lophotrichous.* More than one flagella attached to one pole of the cell.

4. *Peritrichous.* Flagella attached at various points on the sides and at the poles of the cell. Some of these flagella may originate singularly, and some in tufts.

(c) *Spores.* A bacterial spore is the inactive resting or resistant form produced when conditions for growth are unfavorable. It is capable of developing into a new individual when conditions become favorable. Spore formation is not common to all bacteria but is confined largely to the bacilli or rodshaped bacteria. Aerobic sporeformers are found predominantly in the genus *Bacillus.* Anaerobic and microaerophilic spore-forming bacilli are found in the genus *Clostridium.* Both genera are widely distributed in nature. The spore-forming species of bacilli are saprophytic forms found in soil, dust, and water. With the exception of the anthrax bacillus, they are not pathogenic for man under ordinary circumstances. Spore size and location are characteristic in some cases of a specific species; for example, the large terminal spores of *Clostridium tetani* appear as drumstick-like cells. The spore is a very durable structure resistant to heat, dyes, and drying. Many bacterial chemicals cannot penetrate the spore wall. Most spore-formers can be killed by 115°C to 120°C temperatures for 15 to 20 minutes. Sterilization procedures in chapter 4 of this manual outline methods of destroying the microbial spore.

(d) *Inclusion Bodies.* Inclusion bodies are intracellular substances contained in vacuoles or appearing as aggregated masses in the cytoplasm. These substances may be either reserve material for use in the cell or accumulation of wastes produced by the cell (see figure 1–4).

1–5. Colonial Morphology. In the laboratory, a single organism or strain is isolated and plated or seeded on a plate containing solid culture media with various nutrients. The bacteria reproduce millions of times to form an accumulation of progeny called a colony. A colony formed by bacteria through culture exhibits characteristics produced only by that particular organism on the selected medium. The features of colonial morphology that are significant in identification of bacteria are shape, size, texture, color, and growth rate.

a. **Shape.** The shape of a colony may be a clue toward identification of the organism. For example, colonies of *Streptococcus pneumoniae* appear to have small craters on their surfaces, due to autolysis. Some of the colony shapes generally encountered are shown in figure 1–5. To correctly determine the shape of a colony, completely isolate it from adjacent colonies on all of its margins. A stereoscope or magnifying glass is useful when observation for colonial shape is being made. To see the shape of colonies, view them from directly above the culture plate. For the elevation or side view of the growth, view the colonies from the side, using oblique lighting (see figure 1–6).

b. **Size.** The size of colonies, or growth rate, on various media aids in the identification of species. Some of the terms used to describe the size of a colony are "dwarf," "pinpoint," "small," "medium," "large," and "spreading." Colonies increase in size when incubated in excess of 48 hours, due to continual growth of the culture. Distortions or unusual variations in colony size also may be due to natural or induced mutations, or too much or too little moisture in the media or on the surface of the media. Extreme or unusual variations make visual identification difficult; however, if the manufacturer's instructions on media preparation are followed, any media used can probably be eliminated as the cause of any distortion in bacterial growth.

c. **Texture.** Texture of a colony is often a

Figure 1–3. Flagella Arrangement.

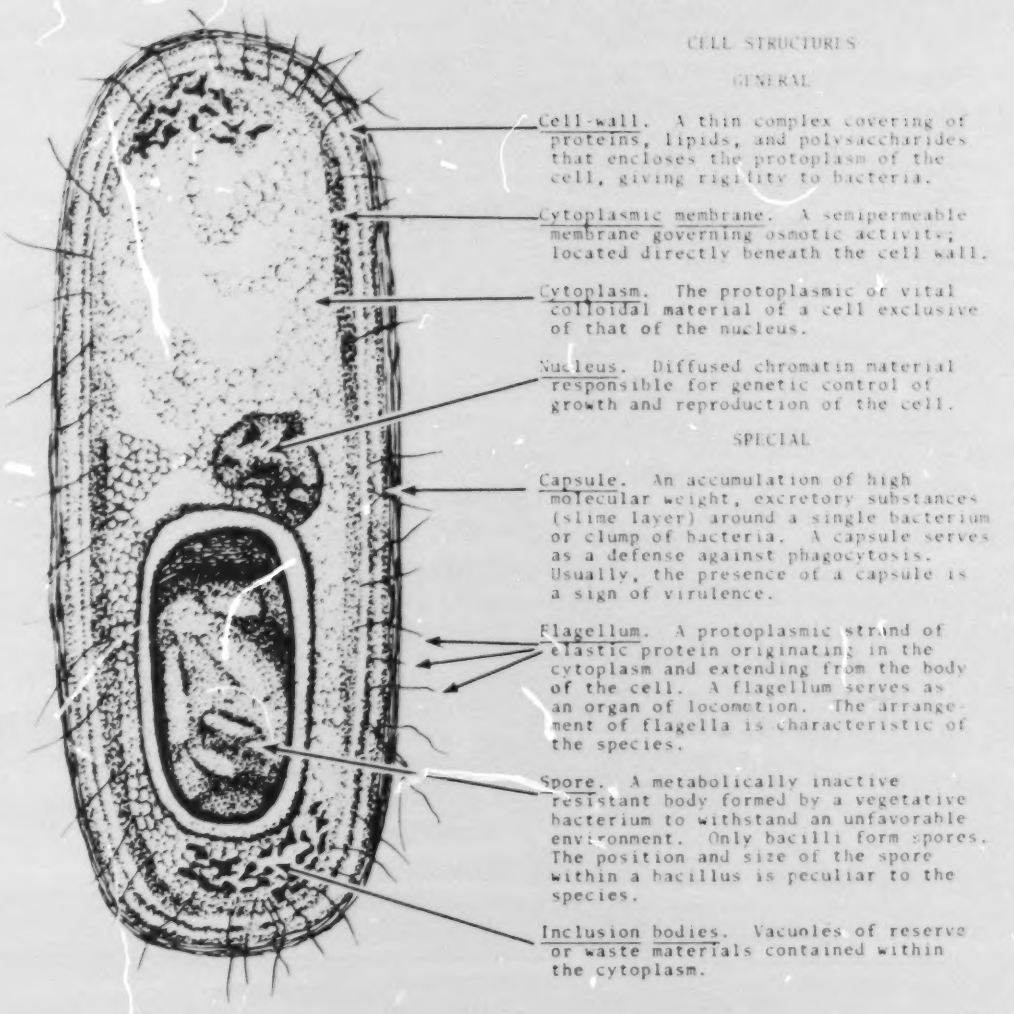

CELL STRUCTURES
GENERAL
Cell-wall. A thin complex covering of proteins, lipids, and polysaccharides that encloses the protoplasm of the cell, giving rigidity to bacteria.
Cytoplasmic membrane. A semipermeable membrane governing osmotic activity; located directly beneath the cell wall.
Cytoplasm. The protoplasmic or vital colloidal material of a cell exclusive of that of the nucleus.
Nucleus. Diffused chromatin material responsible for genetic control of growth and reproduction of the cell.
SPECIAL
Capsule. An accumulation of high molecular weight, excretory substances (slime layer) around a single bacterium or clump of bacteria. A capsule serves as a defense against phagocytosis. Usually, the presence of a capsule is a sign of virulence.
Flagellum. A protoplasmic strand of elastic protein originating in the cytoplasm and extending from the body of the cell. A flagellum serves as an organ of locomotion. The arrangement of flagella is characteristic of the species.
Spore. A metabolically inactive resistant body formed by a vegetative bacterium to withstand an unfavorable environment. Only bacilli form spores. The position and size of the spore within a bacillus is peculiar to the species.
Inclusion bodies. Vacuoles of reserve or waste materials contained within the cytoplasm.

Figure 1–4. Cell Structures.

typical characteristic of the species. Presented here are four basic types of colonies: smooth, rough, mucoid, and L-type.

(1) *Smooth: S Colonies.* The smooth colonies are shiny, moist, elevated, circular colonies similar to the consistency of butter. Under the new classification system, bacteria in Parts 8 and 14 exhibiting S-type colonies are associated with virulence.

(2) *Rough: R Colonies.* Rough colonies appear dry and fragile. They tend to crumble when touched and are difficult to remove with an inoculating needle. Using a needle and syringe also is a good method for removing colonies from culture plates, particularly for anaerobes. In broths or liquid media, rough colonies form flakes and granules and may, in some instances, settle to the bottom of the tube forming a sediment. In the *Streptococcaceae* and *Salmonellae*, the appearance of R colonies indicates a loss of virulence. On the other hand, in the genus *Mycobacterium*, *M. tuberculosis* virulence is associated with the R colony, and the appearance of S-type indicates the loss of virulence. The appearance of R-type colonies in routine cultures can be kept to a minimum by subculturing only S-types, by storing stock cultures in a refrigerator at 4°C, and not subculturing any more than necessary.

(3) *Mucoid M Colonies.* The mucoid colony is essentially an S-type colony of capsulated organisms. It is seen frequently in the Enterobacteriaceae when cultured on rich media. The appearance of the mucoid colony is

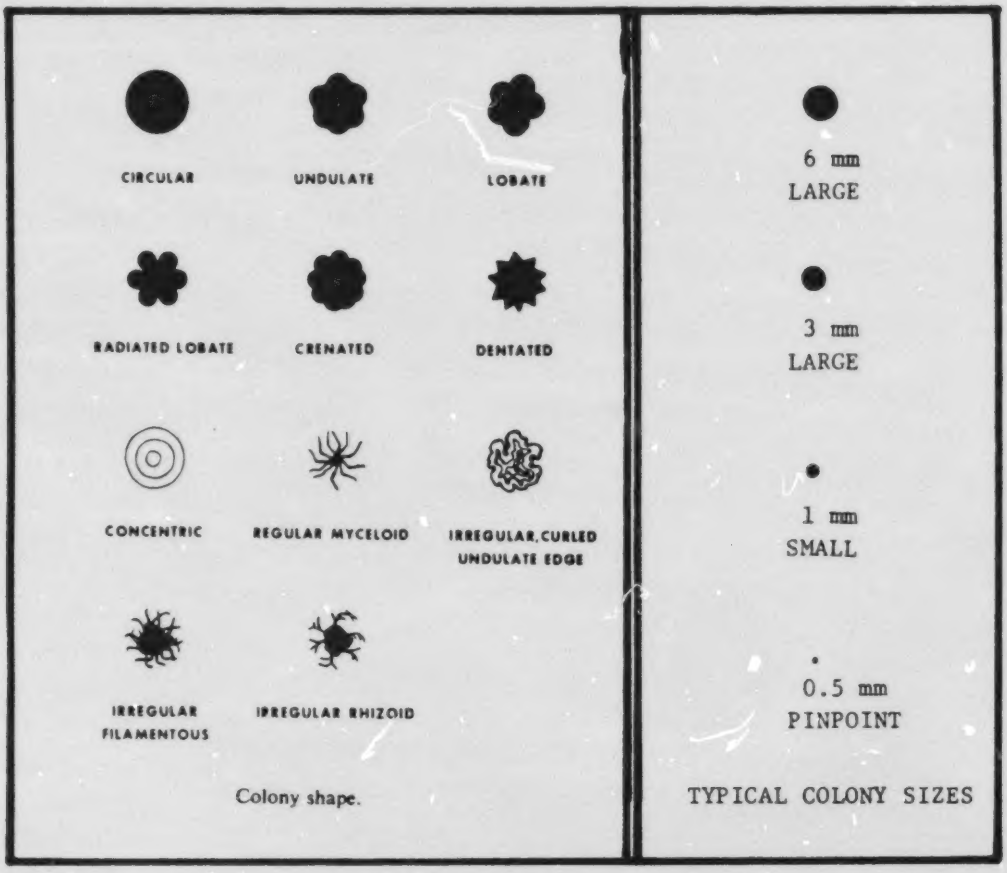


Figure 1–5. Colony Shapes and Typical Colony Sizes.

due to the presence of encapsulated organisms, or a slime produced by the encapsulated organisms. M-type colonies will cling and string out when touched with an inoculating needle. Bacteria which may produce mucoid colonies can produce a scum-like growth in broth media.

d. **Color (Pigment).** Bacterial growth is often naturally pigmented with hues ranging from white to violet. *Serratia marcescens*, for example, produces a red pigment upon standing for a period of time. The pigment produced by most bacteria is not affected by or induced by exposure to light (nonphotochromogenic). However, the family Mycobacteriaceae contains several species of photochromogenic bacteria that develop pigment in the presence of light but not in the dark.

1–6. Biochemical Reactions. Biochemical reactions provide a vast amount of information useful to the bacteriologist in the identification and taxonomic placement of organisms. The family Enterobacteriaceae provides many examples of the use of biochemical reactions in determining the genus and species of a bacteria. As in the case of a culture suspected to be either *Citrobacter* sp. or *Salmonella* sp., these bacteria exhibit many characteristics common to both species. *Citrobacter* sp. also may be confused with *Escherichia* sp. without the differentiating information provided by biochemical reactions. Biochemicals are used widely in the Enterobacteriaceae to identify various species within the genera.

1–7. Gram Staining. Gram staining is a basic step essential to the classification of bacteria. Whether a bacterium is gram-negative or gram-positive is just as important to the identification of that bacterium as information obtained from biochemical testing. Gram staining is discussed in more detail in chapter 3, paragraph 3 of this manual.

1–8. Other Factors:

a. Other factors in the classification of bac-

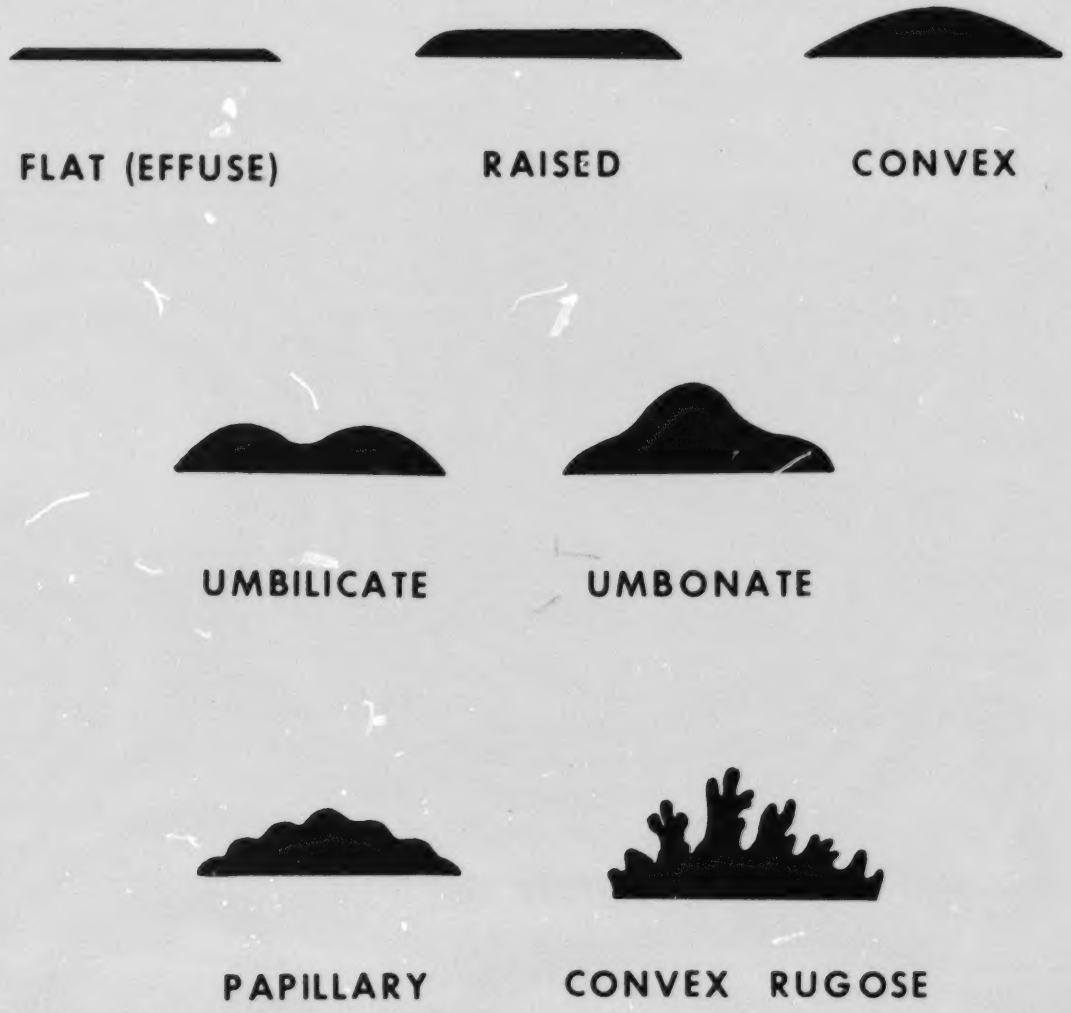


Figure 1–6. Colony Elevation (Sideview).

teria are pathogenic responses and physiological characteristics, such as oxygen, temperature and pH requirements, nutritional requirements, and the response of the organisms to antibacterial substances. Physiological aspects of character, such as whether an organism is an aerobe or anaerobe, also are significant to its identification. Aerobes grow in the presence of oxygen while anaerobes require only minute amounts of oxygen for growth. On the basis of oxygen requirements, bacteria can be classified as obligate aerobes, facultative anaerobes, microaerophiles, and obligate anaerobes.

(1) *Obligate aerobes* grow only in an atmosphere containing oxygen. A culture incubated in an O_2 atmosphere is called an aerobic culture.

(2) *Facultative aerobes* grow in an atmosphere containing oxygen or in an atmosphere lacking oxygen.

(3) *Microaerophiles* grow only in atmospheres containing a minimal amount of oxygen.

(4) *Obligate anaerobes* grow only in an atmosphere completely lacking in oxygen. The quantity and type of gas present other than oxygen affect their growth. For instance, some organisms grow best in atmospheres containing a high concentration of hydrogen (H_2) while others grow best in high concentration of nitrogen (N_2). A culture incubated in an atmosphere lacking oxygen is called an *anaerobic culture.*

b. Temperature and pH requirements of bacteria are also important. In many instances,

the pH at which an organism can survive is critical, as a large number of bacteria are unable to survive at acid pH levels. Also, the existence of many bacteria depends upon the particular organism's optimum temperature for growth. All types of bacteria do not grow equally well at the same temperature. For the most part members of Part 8 (Gram-Negative Facultatively Anaerobic Rods) grow well at 35°C. Most soil and plant bacteria grow at lower temperatures. The occasionally encountered nosocomials also generally grow at lower temperatures (*Pseudomonas, Chromobacterium*, etc). On the basis of temperature requirements, bacteria can be classified as psychrophiles, mesophiles or thermophiles.

(1) *Psychrophiles* tolerate temperatures as low as 4°C. Few pathogens are included, but a notable exception is *Listeria monocytogenes*, the etiologic agent of listerosis, which grows at 4°C. Most psychrophiles are in the "Gram-Negative Chemolithotrophic Bacteria" of Part 12 (Bergey's 8th edition).

(2) *Mesophiles* grow best at temperatures between 30°C to 44°C. Most pathogenic organisms are in this group. A temperature of 35°C is widely used to culture these organisms.

(3) *Thermophiles* grow and flourish at temperatures above 45°C. Notable examples are several *bacillus* species that grow very well at temperatures above 45°C. Several species of bacteria are thermoduric (able to tolerate high temperatures for a period of 24 to 48 hours).

c. Antibacterial substances are useful in the identification of bacteria in agglutination procedures. These procedures are predicated on the fact of antigenic specificity which exists between the bacteria and the antibacterial substances. Typing seras are used in the identification of such organisms as *Escherichia coli* (pathogenic type), *Salmonella sp*, and *Streptococcus pneumoniae*.

1–9. Reproduction of Bacteria. Bacterial cells reproduce by binary fission, splitting at a right angle to their longest axis. The generation time, or time elapsing between formation of a cell and its division, is dependent upon many factors, including the medium, the temperature, the age of culture, and the bacterial strain, genus, or species. The average generation time is 15 to 20 minutes for enteric bacilli and several hours for the tubercle bacilli. Bacterial growth can be viewed in phases of occurrence, based on the inoculation of an adequate liquid medium with an organism and plotting the growth periodically. The typical growth curve exhibits four phases—the lag phase, the log (exponential) phase, the stationary phase, and the period of decline (see figure 1–7).

a. **Lag Phase.** The lag phase begins the instant a bacterium is inoculated upon a medium. At this time a number of biochemical changes take place within viable cells in the absence of cell division. The average cell size increases two to three times its original size. At the end of this period of readjustment to its new environment, the process of reproduction takes place.

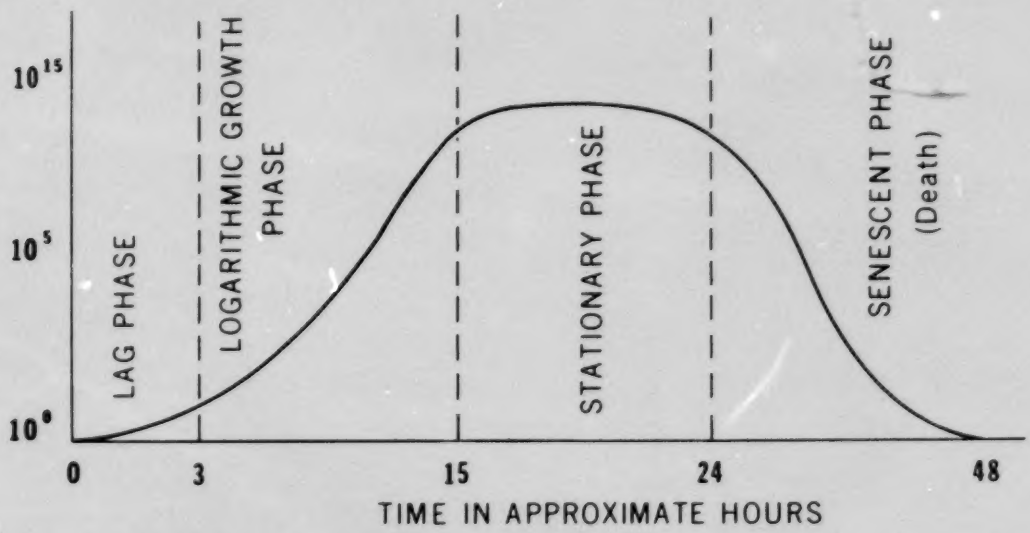


Figure 1–7. Growth Curve (Bacterial).

b. **Exponential Phase.** The exponential phase, or "log phase", exhibits rapid growth and constant generation time determined by the interplay of inherited characteristics of the organism and the environmental conditions.

c. **Stationary Phase.** This is the phase of growth when the bacterial population has reached the point at which the reproduction rate begins to slow down and the death rate equals the generation rate.

d. **Period of Decline (Declining Phase).** During the period of decline the death rate of the bacteria exceeds the generation rate due to: (1) the accumulation of waste products, (2) the exhaustion of available nutrients, and (3) changes in pH. The number of viable bacteria decreases slowly at first, then rapidly until a constant death rate is maintained.

e. **Summary.** Growth, multiplication, and the decline of bacterial growth must be considered as highly dynamic processes involving numerous reactions between the cell and its environment. The first two phenomena represent the establishment of dynamic equilibria which, when fully established, culminate in the steady state noted during the "exponential phase" of reproduction. Any cellular or media changes result in alterations of the steady-state conditions; and if the cell is unable to adjust, cellular activity is inhibited.

1–10. Bacteria and the Environment. The relationship between bacteria and their environment can be shown in three ways: (1) the influence of the environment upon the bacterial cell, (2) the influence of the cell upon its environment, and (3) the interresponse of bacterial organisms within the same environment.

a. **Environment—Bacterial Cell.** The influences of the environment upon the cell may be shown by varying the constituents of the media, changing the cell's surroundings requiring it to survive in a new medium. The more an organism is able to adapt to and utilize different nutrients, the greater is its ability to survive under conditions of change. Variations from the previous "norm" can come about when an organism adapts or mutates to conform to the new environment. Pathogens, for example, are the virulent members of a species; they produce pain and disease. Such bacteria may lose their virulence if grown continuously on a variety of media containing different nutrients. However, if the bacteria is again inoculated to a living animal, the resulting growth will again be a virulent strain of the organism.

b. **Cell Environment.** The influence of the cell upon its environment, physiological effects, may be observed through its pathogenicity. The pathogenicity of an organism is a measure of its ability to cause disease, while virulence is the degree of pathogenicity an organism may possess. Some organisms are highly virulent and can break through the body's defenses easily. Others can invade only with great difficulty and then only when the body has been weakened in some other manner by parasites, a viral infection, or a debilitating disease, such as diabetes. Virulence varies among the various strains of bacteria; some may cause a mild form of disease while others may result in fatality. This should be a word of caution to the laboratory worker; he must be extremely careful in handling all pathogenic as well as potentially pathogenic organisms. This should apply not only to isolates from cases of active disease but also to supposedly mildly virulent bacteria kept in the laboratory as stock cultures. In the case of the latter, passage through a negligent laboratory worker's tissue is a good way to restore the organism to full virulence, giving rise to a spread of infection or disease.

c. **Interresponse of Bacteria.** Little is known concerning the interresponse of bacterial organisms within the same environment, the "normal flora" situations. It is not common to find pure cultures existing in nature; however, it is not impossible. Mixed flora of any population are the products not only of the physical and chemical environment on each individual group of the bacterial population, but also of the biological environment; in this regard, a commensality has been established.

1–11. Organization and Management of the Clinical Laboratory. The work area for handling bacterial specimens should, whenever possible, be isolated from other work areas in the clinical laboratory. Traffic in and out of the department should be kept at a minimum. Ambulatory patients, above all, have no need to visit or pass through such areas. Hand-to-mouth practices, such as eating snacks or nail-biting, should be prohibited in the department.

a. **Essential Observations:**

(1) Lighting should be the best available. A good light source is very important when handling microscopic organisms.

(2) The department should be kept clean at all times, cutting down on the possibility of contamination or infection while working in the area.

(3) Ventilation should include filters to

clean any air entering the area to prevent airborne microorganisms from contaminating cultures and workbenches. Filters also should be used to prevent possible spread of any microorganisms which may result from cultures being carelessly handled in the department.

(4) Equipment should be kept in good working condition, and checked at regular intervals. A log containing this information should be maintained and used as part of a quality control program.

b. **Quality Control.** A system should be devised whereby all work produced in the Bacteriology Department is of the same high quality on a day-to-day basis. Checks and balances should be devised to allow the detection of any variation in the department which may affect the quality of work. Maintaining stock cultures of frequently isolated organisms gives an excellent source of bacteria to use for checking media for consistency of reactions, particularly the differential and selective media. Stock organisms may provide a means of checking the consistency of media reactions in the quality control program. Table 1-2 shows some of the media reactions which can be checked using ATCC strains of *Staphylococcus aureus* and *Escherichia coli* and other bacteria commonly used. Other organisms also may be obtained from the ATCC (American Type Culture Collection, 12301 Parklawn Drive, Rockville MD, 20852) or other sources listed in the *World Directory of Collection of Cultures of Microorganisms* (Martin and Skerman, editors), Wiley-interscience, 1972. Quality control in bacteriology also may be provided through proficiency testing obtained from sources such as the Center for Disease Control (CDC) in Atlanta, GA, state public health laboratories, or larger military regional hospitals at no charge to the requesting facility. Commercially there are many sources from which proficiency surveys may be obtained; one such source is the *College of American Pathologists* (CAP) Survey. Checks can be made on media preparations to insure the steps required for proper usage as a culture medium. Disc expiration dates should be checked on a regular basis to assure sensitivities are being reported accurately. Maintaining an incubator temperature of 35° ± 1° at all times is also important to the success of any quality control program.

c. **Handling Infectious Material.** The technician should always be aware of the possibility of infection when working with and around microorganisms. A program of good, sensible cleanliness will provide a measure of protection to the technician against accidental contamination, but sometimes this is not enough. In most cases the technician cannot control the manner in which clinical cultures are submitted for processing by outpatients or ward personnel. The technician can, however, be on the lookout for leaking containers, a sure source of contamination to personnel. Ward personnel should be instructed, whenever possible, as to proper procedures in the collection and submission of clinical specimens. Caution must be exercised when handling materials to be examined for the presence of *M. tuberculosis*. Whenever possible, a ventilated hood should be used for processing microorganisms which may become airborne hazards to laboratory personnel (for example, *Coccidioides immitis*).

(1) *Handling and Sterilization of Contaminated Containers:*

(a) Make sure that the lids of urine and sputum cups are firmly snapped down to avoid spillage.

(b) Be sure to autoclave containers of urine or sputum prior to disposing of them. The urine is either poured down the drain or a small amount of concentrated phenol can be added to the specimens and allowed to stand for about 10 minutes before discarding.

(c) Specimens are sometimes received in glass tubes; the caps should be carefully checked for tightness and signs of leakage. Material contained in the tube may have seeped onto the outer surface of the cap or the tube. If there are indications of surface contamination, the tube should be capped securely taking care not to get the material on the hands. A solution of 5 percent phenol is suggested for decontaminating the surface for safer handling. Hold the unused portion of the original specimen until accurate results are obtained before autoclaving to discard it.

(d) Stool specimens are often submitted for the detection of enteric pathogens on foodhandler examinations. These specimens should be placed in a disposable plastic bag and placed in an incinerator at the end of the workday.

(2) *Bench Precautions:*

(a) Benchwork should be performed on a nonabsorbent surface to prevent the possibility of contamination by organisms harbored in the cracks and crevices of the bench surface. Smooth nonabsorbent surfaces can be rendered free of bacteria for short periods of time with a cleaning solution. Such surfaces are therefore the preferred type of material

Table 1-2. Quality Control For Some Commonly Used Media.

	Blood agar (sheep blood)	EMB agar	SS	XLD	Mycosel agar	CTA	Thayer Martin medium	Chocolate agar with supplement A or B	Chocolate agar without supplement	Mannitol salt agar	TSI agar	Motility
Escherichia coli ATCC 25923	+ hemolysis	+		+			-			-	+	
Staphylococcus ATCC 25922	+ hemolysis (beta)	-		-	-		-			+		
Neisseria gonorrhoeae ATCC 13077						+	+	+	±			
Neisseria Meningitidis ATCC 19424						+	+	+				
Salmonella sp ATCC 13314		+	+	+								
Candida sp					+							

for bench surface construction.

(b) Clean hands are very important to the technician when working with and around bacteria. Contamination is the prime concern in and out of the department. The hands should be washed before leaving for another department, and prior to returning from an outside area or department.

(c) When liquids require measurement with a pipette, always use a suction apparatus. Never aspirate any bacterial specimens or reagents by mouth, under any circumstances.

(d) Dispose of contaminated pipettes by first placing them in a disinfectant until autoclaving can be accomplished. If reusable pipettes are used, they should be placed in the disinfecting solution, rinsed, and autoclaved before washing for reuse.

(e) At the end of the work day the bench area should be washed down with a disinfectant as an added precaution against possible spread of harmful bacteria.

(3) *Avoidance of Airborne Contaminants.* The use of the inoculating loop is basic to the performance of many procedures in bacteriology. The loop also is a possible source of airborne bacteria. Airborne particles may be inhaled or become seeded on a nearby surface and develop into an infection or a source of contamination. Place loops into a flame to remove any organisms present before and after each inoculation procedure is performed. When a loopful of specimen is introduced into an open flame, the material may "pop" or "splatter" and become airborne due to sudden heat application. The reverse of this situation—that is, placing a hot loop into a bacterial solution—also may cause particles to become airborne.

(4) *What to Do When an Accident Occurs.* Spillage of infectious materials should be taken care of immediately to prevent the spread of a possible pathogenic organism. If a specimen containing bacteria is accidentally broken or spilled, a strong disinfectant should be applied to the area and allowed to stand about 5 minutes. A sponge or mop may be used to remove the mixture from the surface area and placed in a bucket until it can be properly disposed of in a safe manner. The liquid may be autoclaved or poured into a drainage system if rendered safe with a bactericidal agent. After localized contact with infectious substances, the skin should be washed with a germicidal soap, then rinsed with a copious amount of water. If clothing is involved, it should be removed as soon as possible and decontaminated by autoclaving or use of chemicals. If bacteria gets into a laceration, the following steps should be taken:

(a) If the wound is not bleeding, induce blood flow, to flush bacteria from the site.

(b) Cleanse the wound immediately with running water or clean water from a laboratory washbottle.

(c) Wrap the wound with a sterile piece of cloth or any clean material available.

(d) Contact your supervisor and report the emergency immediately.

Chapter 2

BACTERIOLOGY SPECIMENS

2–1. Introduction. The proper collection and processing of a bacteriological specimen is important to the eventual isolation and identification of bacterial agents of disease. This chapter outlines general and specific rules for collecting and processing bacteriological specimens for the clinical laboratory. The collection of samples for viral studies has been added to this chapter in paragraph 2–5, accompanied by a table on sample handling for easy reference.

2–2. General Considerations. Certain aspects of specimen collection which aid in quality control or the control of contaminants interfering with isolation or identification of "pure cultures" are listed below:

a. Instruments, containers, and other equipment coming in direct contact with specimens must be STERILE. There are few exceptions to this condition.

b. Specimens for culture must not come in contact with chemicals, disinfectants, and germicides.

c. Obtain specimens for culture before the patient receives antibacterial therapy, if possible. If therapy has been started, indicate this fact on the current bacteriology request forms.

d. Label specimens properly, giving the patient's full name, physician, ward number, register number, bed number, time, and the date, and deliver them to the clinical laboratory immediately following collection.

e. Establish a receiving area so that bacteriological specimens containing possible pathogens will not be left in several scattered areas within the laboratory facility.

f. Establish provisions for storing or processing specimens after normal operating hours.

g. Avoid contamination of culture specimens before making smears or performing special test, whenever possible.

h. Inoculate specimens to media immediately after receiving them in the clinical laboratory.

2–3. Specimen Collection and Submission. Improper collection of specimens very frequently results in failure to isolate and identify suspected pathogens. Methods of collecting the most common specimens are given below. Also consult table 2–1. (Flow charts for specimen are presented in this chapter.)

Table 2–1. Collecting and Processing of Bacteriological Specimens for Shipment.

Specimen	*Processing for Shipment**	*Remarks*
Ascitic fluid for bacterial and fungal culture.	Place aseptically in sterile containers without preservative.	Where tuberculosis is suspected, see directions for processing specimens from suspected tuberculous patients.
Bacteria, pure cultures of, for identification, confirmation and sensitivity tests.	Grow in pure culture on beef extract or beef infusion agar in screw-cap tubes. Prior to shipment, tighten screw-caps and secure with adhesive tape or by dipping in melted paraffin.	Plant subcultures on agar slants and incubate 12 to 18 hours. A duplicate of each culture should be retained in the laboratory of origin until a final report is rendered.
Blood or urine for culture.	Collect 3 ml of blood aseptically before serum, chemo- or antibiotic therapy and transfer to rubber-stoppered bottle containing hemoglobin-tryptosephosphate broth.	Prior to shipment, incubate specimens for 12 to 24 hours.
Serum for bacterial and cold agglutinins, antibiotic levels, and complement-fixation tests.	Collect blood aseptically in sterile evacuated tubes, filling to capacity.	Allow firm clot to form before packing for shipment. Blood for cold agglutination tests must not be refrigerated until cells are separated from serum.
Cerebrospinal fluid for bacterial culture, and antibiotic and sulfonamide level.	A minimum of 3 ml of fluid is collected aseptically and inoculated into hemoglobin-tryptosephosphate broth.	Where tuberculosis is suspected, see directions for processing such specimens. Retain broth culture under incubation 12 to 24 hours prior to shipment.

* For more information concerning shipping containers, consult AFM 160–28, *Methods of Preparing Pathologic Specimens for Storage and Shipment.*

Table 2–1. (Continued).

Specimen	*Processing for Shipment*	*Remarks*
Throat cultures for C. *diphtheriae*.	Inoculate Loeffler's serum slant with a fresh throat swab.	Mail specimen immediately.
Exudates for bacterial and fungal cultures.	Place swabs aseptically in sterile screw-capped containers without preservative.	
Fecal specimens for bacterial culture (except tuberculosis).	Emulsify a 5-gm sample aseptically in a sterile screw-capped vessel containing one part C.P. glycerin and three parts 0.6 saline solution.**	In food poisoning outbreaks, feces should be collected from patients with diarrhea and all foodhandlers associated with the outbreak.
Swabs for culture from open lesions.	Swab lesion with broth-soaked swab and place aseptically in sterile screw-capped test tubes.	
Tuberculosis, specimens for smear, culture and animal inoculation (other than sputum and gastric contents).	Collect fluids, tissue, or feces in suitable sized sterile containers without preservatives.	Specimens include ascitic fluid, cerebrospinal fluid, joint fluid, pleural fluid, pus, urine, lymph node, and other tissue and feces.
Tuberculosis, sputum specimens.	Collect all sputum coughed up on three consecutive mornings after first having patient wash teeth and rinse mouth thoroughly with boiled water. Place entire 3-day specimen in a sterile wide-mouthed screw-capped volume of 20 percent trisodium phosphate.	During collection, keep jars out of direct sunlight or the tubercle bacillus will be rendered nonviable and, therefore, unsuitable for culture or animal inoculation. *Do not use cardboard sputum cups for collection.*
Tuberculosis, gastric contents.	Through sterile stomach lavage tub introduce 200-300 ml of sterile saline solution into fasting stomach. Evacuate stomach and place entire specimen in suitable sterile container.	Prior to placing specimen in shipping container, neutralize it to litmus with 20 percent sodium bicarbonate.
Specimens for bacteriological culture.	(1) Any suspected canned foods should be shipped unopened in original container. (2) Well mixed representative samples of suspected prepared food should be placed aseptically in suitable containers without preservative. (3) Vomitus from all cases in which vomiting is present should be placed aseptically in suitable sized containers without preservative. (4) Swabs from lesions taken from foodhandlers should be placed in nutrient broth using aseptic technique and transferred to sterile screw-capped est tubes.	Complete epidemiological history should accompany any specimens taken from food poisoning outbreaks.
Joint or other body fluids for bacterial and fungal cultures.	Collect joint and other body fluids and transfer aseptically to suitable sized sterile container without preservative. Plant fluids for mycological culture on Sabouraud's media, or place in sterile screw-capped containers without preservative.	
Sputum for pneumococcus culture or typing.	Collect aseptically before serum, chemo—or antibiotic therapy is started and place in sterile container without preservative.	

** If shigellosis is suspected, immediately freeze the specimen in a solution of alcohol and dry ice. Pack the frozen specimen in cardboard carton with dry ice for shipment.

a. **Blood:**

(1) The ideal method of collecting a specimen of blood for culture is to use a sterile, disposable blood culture set. The set consists of a plastic tube with a needle on each end and a collecting bottle (B-D Vacutainer). One needle is inserted into the patient's arm and the other through the diaphragm-stopper of a blood culture bottle. Blood is allowed to pass directly from the patient to the bottle and chances of contamination are reduced. Five to 10 milliliters of blood is the amount recommended per bottle of medium inoculated.

(2) Blood also can be collected with a sterile needle and syringe. Use a 10-ml syringe and collect the sample for culture.

(3) With either collection method, special care must be given to disinfection of the area selected for venipuncture. One procedure for cleansing is to swab and wipe the area first with green soap using a sterile gauze, and then with 70 percent isopropyl alcohol; follow this by swabbing with tincture of iodine (3.5 percent). Wipe the iodine from the site with alcohol, and repeat the iodine followed by the alcohol. Apply each solution, and allow it to dry before applying the next. Make the venipuncture immediately upon completion of the cleansing procedure.

(4) Failure to isolate organisms from blood cultures is sometimes due to collection of a specimen at the wrong time. In the case of patients having chills and fever, collect the specimen just before or during the occurrence of the chills and fever.

(5) Take four specimens per request for culture at regularly spaced intervals over a 36- to 48-hour period. Incubate two specimens aerobically and the other two anaerobically. (See flowchart, figure 2–1.)

b. **Throat:**

(1) To take throat culture specimens, use a sterile calcium alginate wool or a Dacron-tipped swab. Cotton-tipped swabs are not recommended because of the inhibitory effects of substances found in cotton.

(2) Use a tongue depressor to hold down the tongue and insert the swab into the far back-throat. Specimens for throat cultures should be taken from the rear throat area, or near the tonsils, not the walls of the mouth. (See figure 2–2.)

(3) Many throat pathogens die rapidly. The specimen must be prevented from drying and should be inoculated on culture media as soon as possible.

(4) If throat cultures cannot be plated immediately, the specimen should be placed in a transport or holding medium, if necessary. The newer culture swabs contain an ampule of preservative which will sustain the bacteria present for at least 72 hours.

(5) Some authorities recommend immediately placing the swab used to take the culture in a sterile tube containing 1 to 2 ml of sterile trypticase soy or tryptose soy broth.

(6) Portions of the applicator touched by the collector's fingers should be broken off prior to placing the applicator in a sterile tube for transport. This is accomplished by placing the applicator three-fourths of the way into the tube and sharply breaking the applicator.

(7) Prepared sterile applicators in disposable tubes specially designed for collecting cultures are available through Federal supply channels. The stick is attached to the cap of the tube so that the applicator is never touched (see figure 2–3). Culturettes are also available, containing a preservative solution (Stuart's holding medium) in an ampule (see figure 2–3). The ampule is crushed upon collection of the culture, allowing for slight (up to 72 hours) delay prior to plating.

(8) Throat specimens are occasionally taken by means of gargling. The patient is given three successive 5 to 10 ml aliquots of sterile buffered water and allowed to expectorate into a widemouth jar. The gargling should not be a mere swishing about in the mouth. It should be a thorough gargle with the HEAD HELD BACK.

(9) A West swab (figure 2–4) is advantageous for use on small children. It is made by tipping a helix of thin flexible copper or nichrome wire with calcium alginate wool or Dacron.

(10) A flowchart for processing throat cultures and similar specimens is shown in figure 2–5.

c. **Nasopharyngeal:**

(1) Collect specimens with calcium alginate wool or Dacron-tipped applicators.

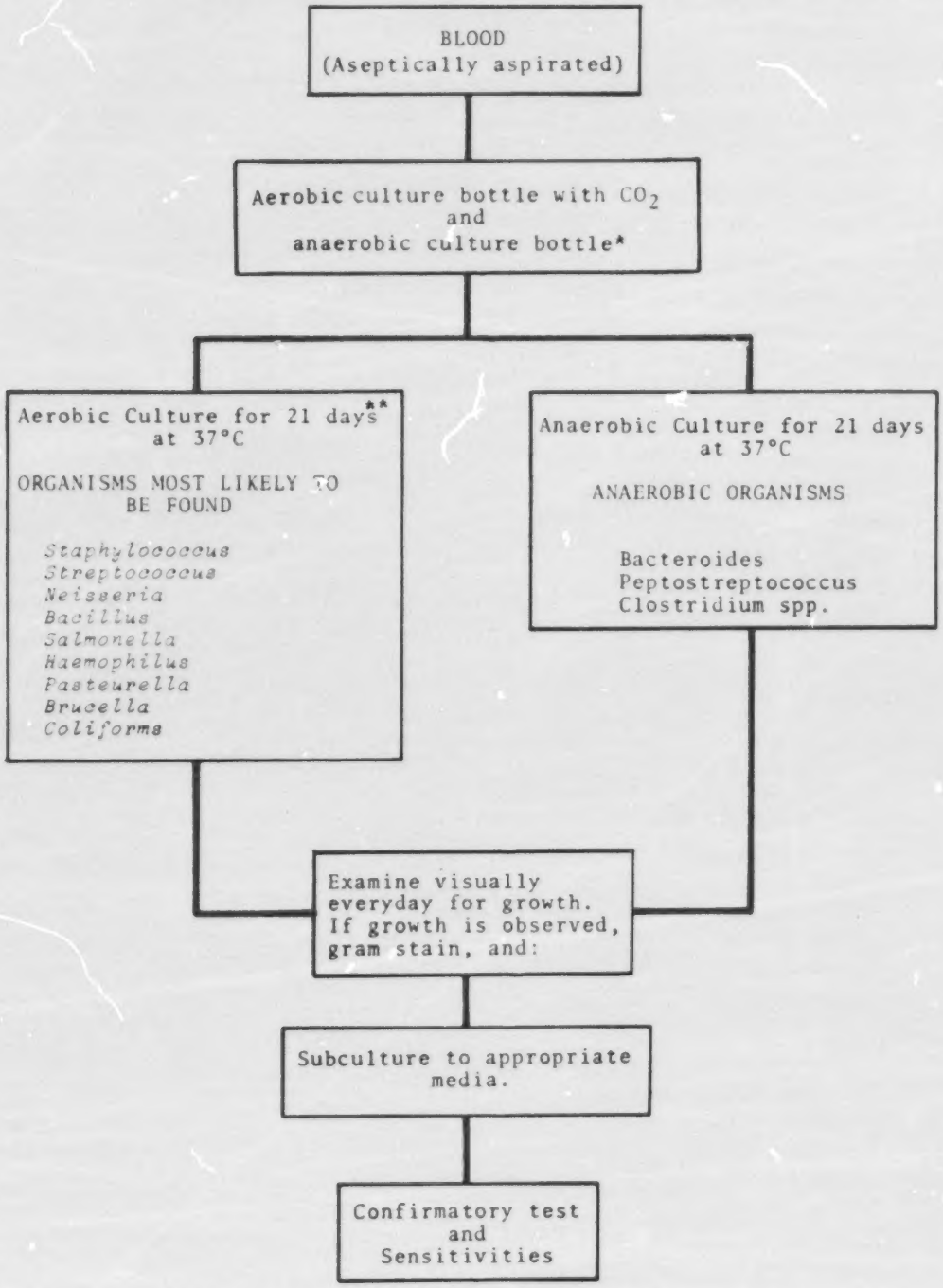
(2) West swabs prepared as above are preferred because the specimen can be taken from the deep rear area of the nasopharynx.

d. **Sputum:**

(1) Sputum is thick, puslike discharge from the bronchial passages and should not be confused with saliva from the mouth.

(2) Collect the specimen by getting the patient to cough deeply several times, expectorating 5 to 10 ml of sputum into a sterile widemouth jar.

(3) Specimens from patients suspected of



*"Blind Subculture" at either 48 or 72 hour period.

**With the exception of Brucella, 14 days has been shown to be sufficient incubation time for blood cultures. 21 days rules out Brucella species.

Figure 2–1. Technique for Blood Cultures.

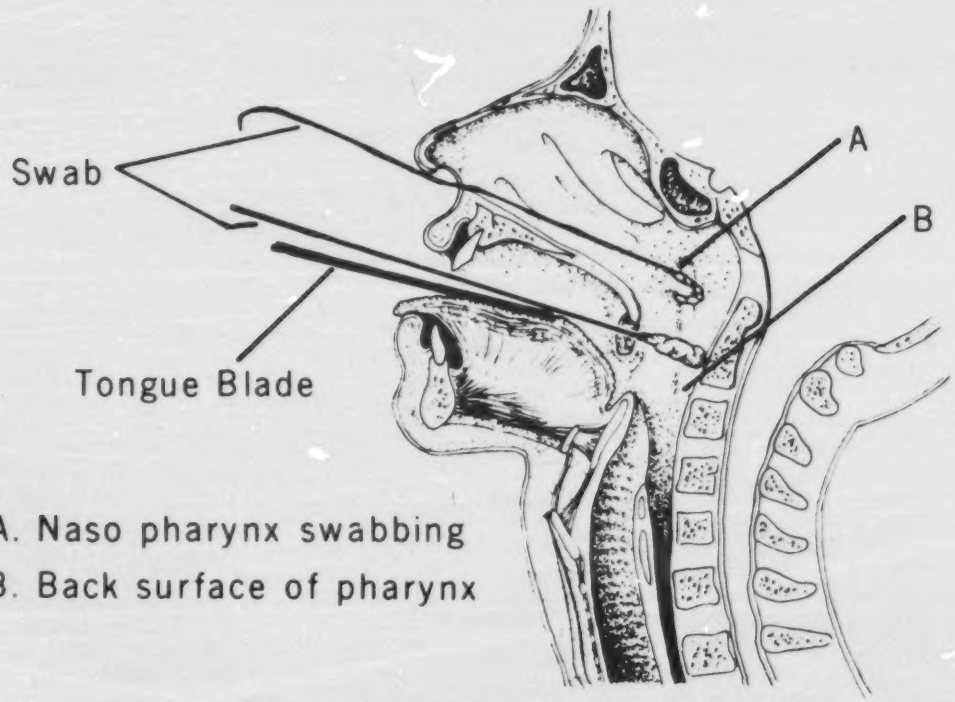


Figure 2–2. Obtaining Specimen for Throat Culture.

having tuberculosis should be the first specimens of the morning. Exercise care with these specimens because *Mycobacterium tuberculosis* is a dangerous and highly infective pathogen.

(4) A flowchart for processing sputum and associated specimens is shown in figure 2–6.

e. **Exudates.** An exudate is a drainage or discharge and can be obtained from wounds, boils, ears, eyes, mastoid infections, chancres, or skin rashes. Collect as follows:

(1) Most exudates can be collected on sterile tipped applicators. When available, allow the exudate to saturate the swab.

(2) Exudate from chancres in suspected syphilis should be collected in a small (tuberculin or 2 ml) syringe.

(3) Sterile wire loops can be used. Be sure the loop is cool before touching the patient.

(4) In collecting urethral discharge in suspected gonorrhea infections, be tactful and discreet; arrange a private collecting place.

(5) Occasionally in gonorrhea infections, a discharge is difficult to obtain. In this instance get the patient to apply a firm, downward massage to the penis. A loop is better than a swab if the discharge is light.

(6) If specimens are taken from deep wounds that might harbor anaerobic organisms, the specimen should not be exposed to atmospheric oxygen any more than necessary, and culture should be performed immediately.

(7) A flowchart for processing exudates is shown in figure 2–7.

f. **Urine.** Urine specimens may be either normally passed specimens or specimens taken by means of a catheter. Studies have shown that obtaining catheterized specimens does not significantly affect the recovery of most pathogens found in urine. Catheterization is, however, of value in a few unusual urological conditions. Urine samples are collected as follows:

(1) Routine urine specimens for culture should be collected in sterile widemouth jars. Collect 30 to 60 ml of early-morning, midstream urine. Three early "clean-catch" or catherized specimens are also recommended for TB studies. If the specimen is not processed immediately, store it in the refrigerator to maintain the relative quantities of organisms present in the specimen at the time of collection.

(2) Urines are occasionally collected as two-glass or three-glass specimens. To collect these specimens, the patient is given sterile

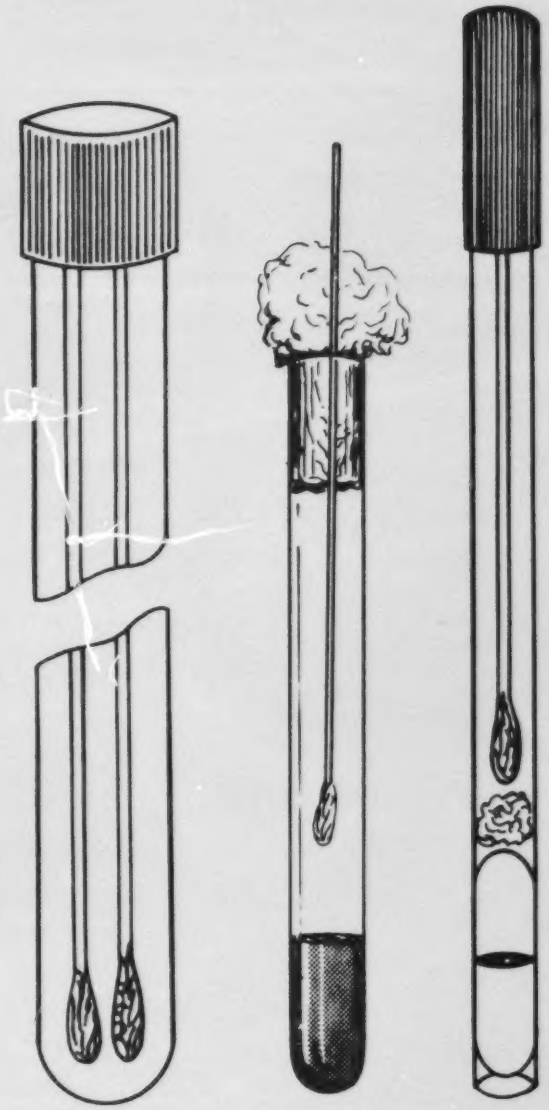


Figure 2–3. Culture Tubes (3).

widemouth jars labeled 1–2 or 1–2–3, and instructed to put the first urine passed in jar number one, the next portion in jar number two, and the last portion in jar number three This procedure is of value in pinpointing the region of urinary tract harboring bacteria. (urethral, bladder, or kidney).

(3) Urine can be collected over a 24-hour period for routine culture. The samples passed should collectively be stored in a 2- or 4-liter jug kept stored in a refrigerator.

(4) Urine specimens taken in cases of suspected tuberculosis are collected over a 24-hour period and stored as above. The urine should be processed as soon as the collection is finished. Tubercle bacilli die if left standing in urine for a great length of time.

(5) Specimen collection requires that the patient be instructed concerning possible contamination of samples in the process of collection. A procedure for collection of specimens for examination should therefore be posted for patients' use during collection.

(a) Male patients should be instructed to thoroughly clean the foreskin area of the penis with a mild cleansing agent and rinse with a sterile saline solution using sterile gauze. Following the cleaning procedure, the specimen is then collected in a sterile container, the lid replaced, and the specimen given to the technician for processing.

(b) Female patients may find it difficult to cleanse themselves free of bacteria for urine collection. A sterile wide mouth jar with lid should be provided for the specimen. The patient should be given instructions concerning cleansing of the vaginal area prior to collection. A nurse is often asked to instruct female patients, and explicit instructions are placed in the collecting area for their referral. Essentially, the patient is asked to clean the vaginal area (labia majora, labia minora) with mild soap and wipe with sterile saline using sterile gauze; the labia is held apart preventing contact with the passed specimen.

(c) Catheterized specimens should be collected only when the patient is unable to voluntarily collect a specimen. Catheterization procedures are performed by ward personnel or in the emergency room when patients can not void. Specimens for processing anaerobically require suprapubic aspiration of urine, that is, by using a syringe and needle to collect urine from the bladder through the abdominal wall.

(6) Processing. After urine is collected it should be immediately inoculated to the appropriate media (blood agar, MacConkey's) and incubated at 35°C for 24 hours or stored in the refrigerator. Centrifugation of specimens in sterile test tubes may be used to concentrate bacteria, if desired. However, in most instances growth is obtained from uncentrifuged samples. Unspun specimens are used for colony counts.

(7) Colony Counts:

(a) Urine colony counts are used to determine the number of organisms in a milliliter of urine as an indication of the severity of infection. A calibrated loop is introduced into the specimen, inoculated to blood agar and

Figure 2–4. West Swab.

spread over the media surface evenly. Spreading the inoculum evenly is important because colonies can be counted easier when isolated and not grown in a bunched fashion.

(b) Loops used in colony counts are calibrated to afford easy and accurate enumeration of bacterial growth. The calibrated loop is prepared to remove 1 one-thousandth of a milliliter of urine from the specimen given a count of 1000 colonies per colony grown in the culture. On the basis of the growth obtained, counts of 100,000 organisms per ml are considered medically significant in an infection, but lower counts also may be important to the physician in treatment of the patient. Report the results of a colony count as follows:

1. Less than 1,000 bacteria per milliliter.

2. Between 1,000 and 100,000 bacteria per milliliter.

3. Greater than 100,000 bacteria per milliliter.

(c) Counts less than 1,000 bacteria per milliliter are generally regarded as a result of contamination; however, there is no definite number of bacteria which distinguishes between an infected and contaminated specimen. Treatment of a patient is only determined by the physician with respect to the overall medical data; hence, the technician should report the colony count even when the number is low.

(8) A flowchart for processing urine specimens is shown in figure 2–8.

g. **Body Fluids:**

(1) Body fluids are collected by the physician. They are routinely collected by means of sterile syringes, placed in sterile tubes, and sent to the laboratory for study. By using a syringe for collection of these fluids the technician is allowed an excellent opportunity to recover anaerobic organisms. The specimen should either be plated immediately to an oxygen-free media or sealed in an oxygen-free atmosphere for a short time until it can be processed. As a rule, however, all anaerobic cultures are best performed upon receipt.

(2) Source of the fluid specimen can be an important factor in how the specimen is handled and the type of medium inoculated. Fluid specimens are named according to the part of the body from which they were taken, as for example:

(a) Ascites—fluid in the peritoneal cavity. Peritoneal fluid is another name used to identify the specimen.

(b) Pericardial—fluid from the membranous sac around the heart.

(c) Cerebrospinal—fluid from the intrameningeal space.

(d) Synovial—fluid from an inflamed joint, such as an ankle, wrist, knee, etc.

(e) Pleural—fluid from the chest cavity.

(3) Many fluid specimens will coagulate (clot) if left standing. Therefore, processing should be immediate.

(4) Fluid specimens occasionally contain highly infective pathogens and should be handled with care to prevent the spread of infection.

(5) Fluid specimens are placed on prereduced media, freshly prepared, which has been stored anaerobically not longer than 72 hours. Immediately place inoculated plates or

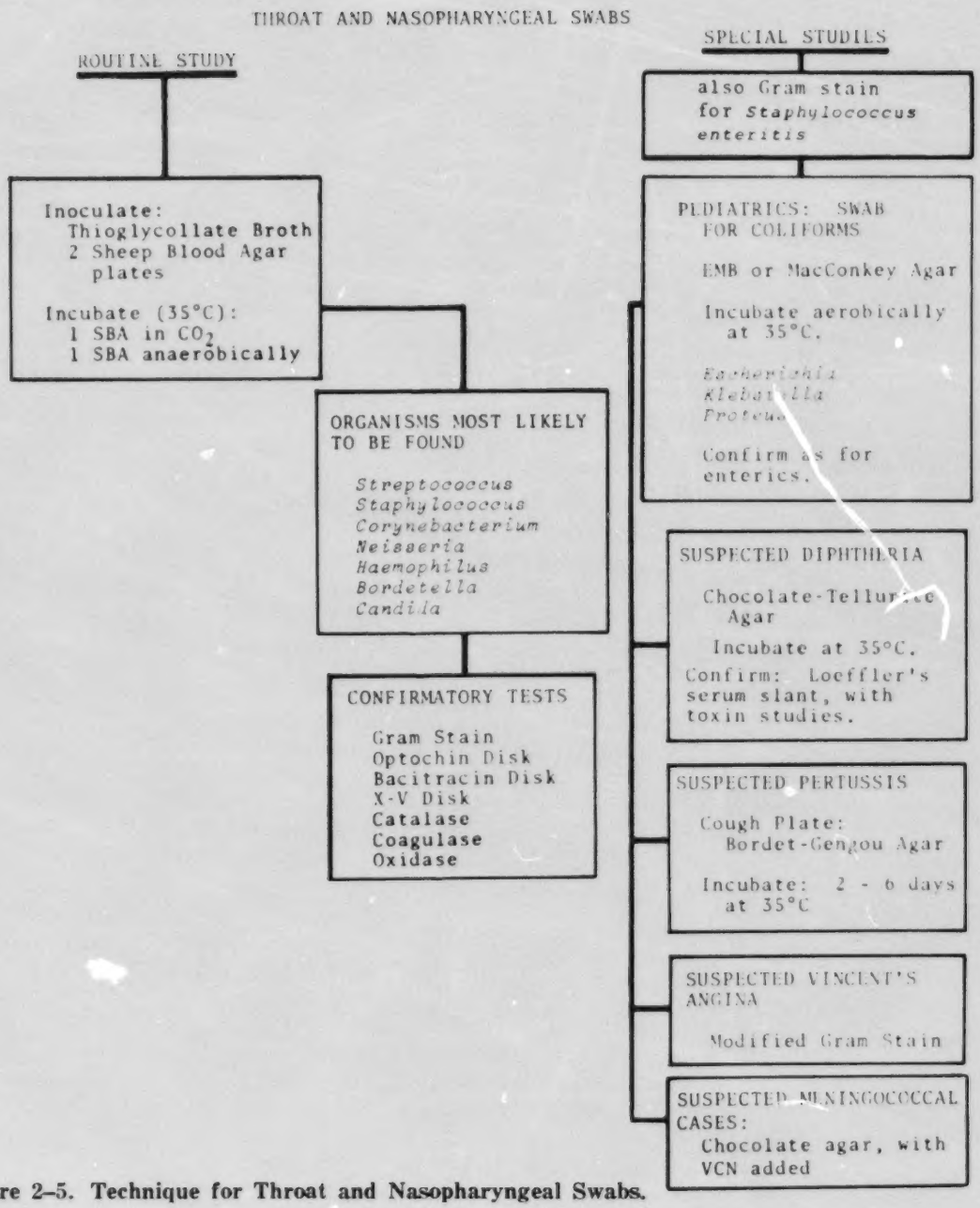


Figure 2–5. Technique for Throat and Nasopharyngeal Swabs.

tubed media under anaerobic conditions for a minimum of 48 hours.

(6) Flowcharts for processing cerebrospinal and other body fluids are shown in figures 2–9 and 2–10.

h. **Fecal Specimens.** Fecal specimens are about the only type of specimen not collected in sterile containers. They are usually collected in waxed cardboard containers (available through Federal supply channels). Collect as follows:

(1) Loose, watery stools that occur in diarrhea are the most promising specimens. If the specimen cannot be processed immediately, transfer it to a suitable transport or holding medium or add equal volumes of 0.003M phos-

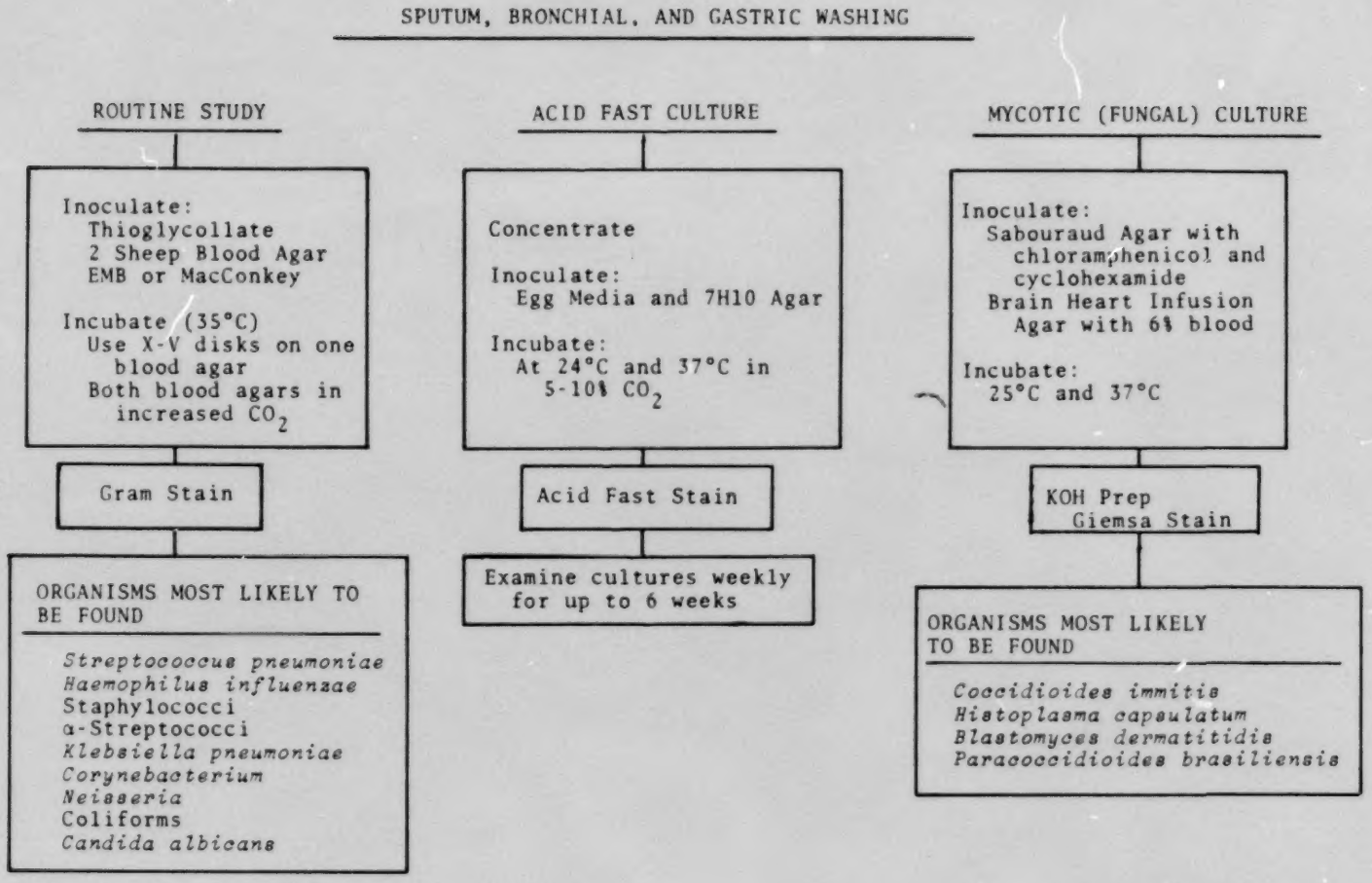


Figure 2–6. Technique for Sputum, Bronchial, and Gastric Washing.

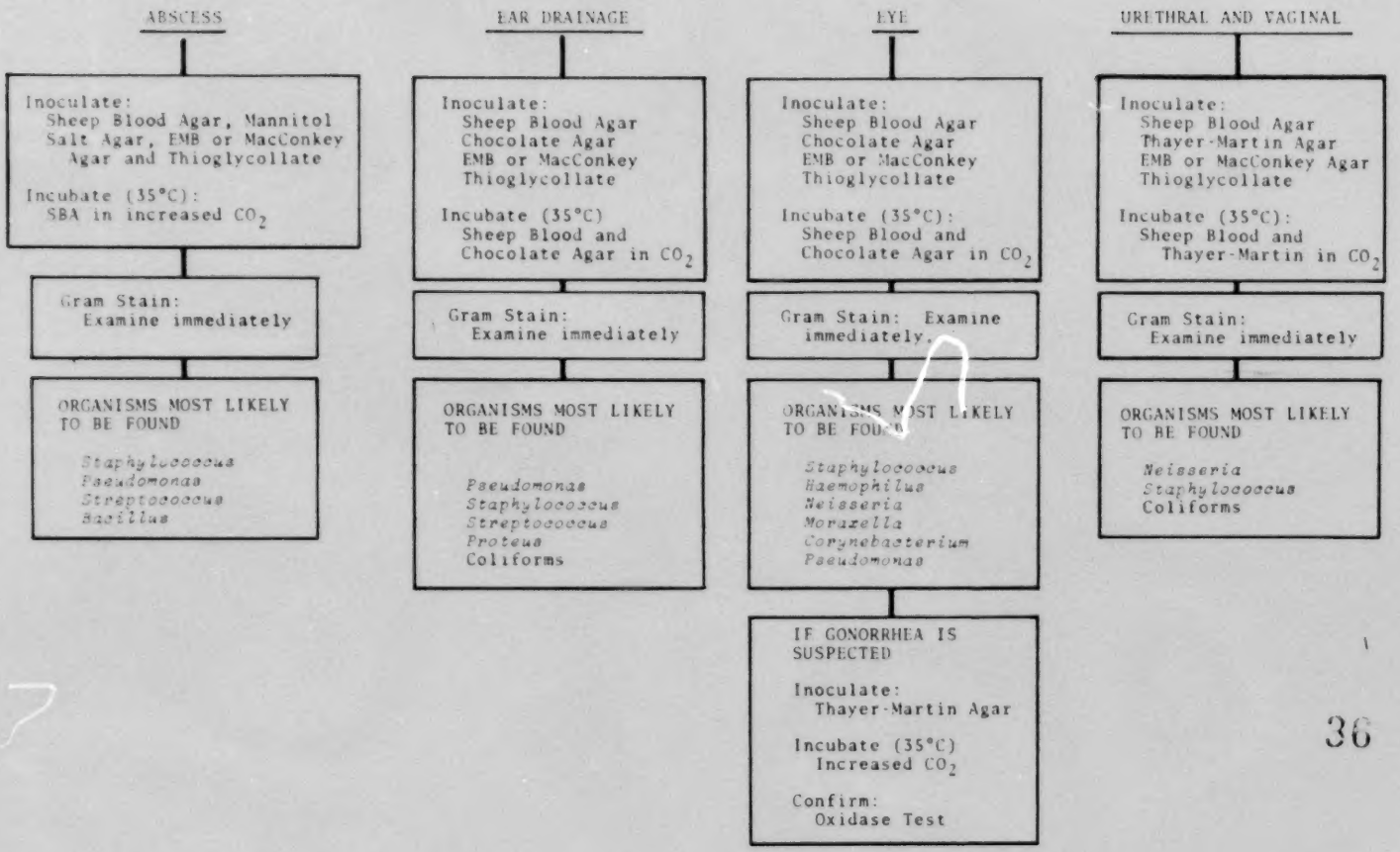


Figure 2–7. Technique for Exudates.

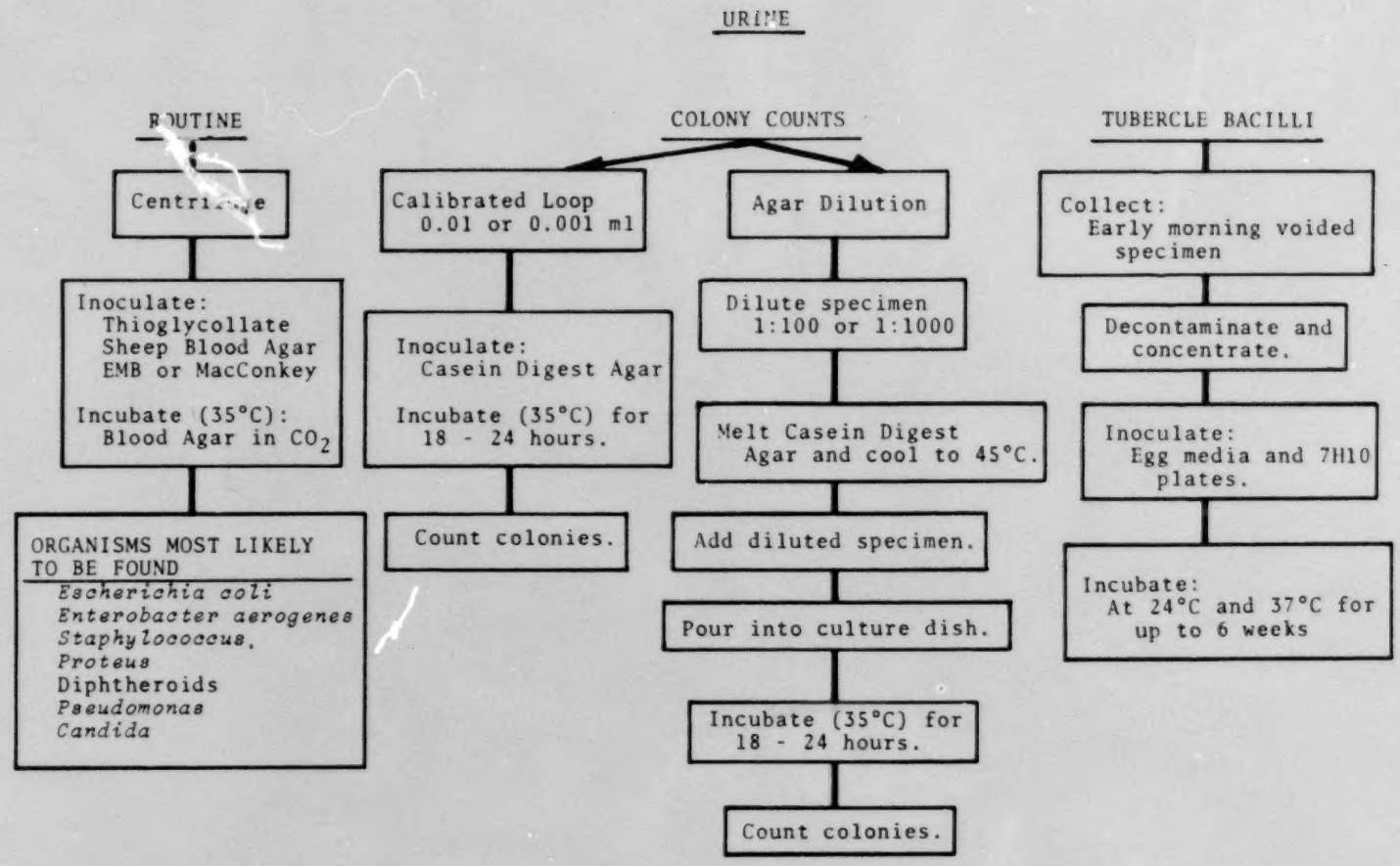


Figure 2-8. Technique for Urine.

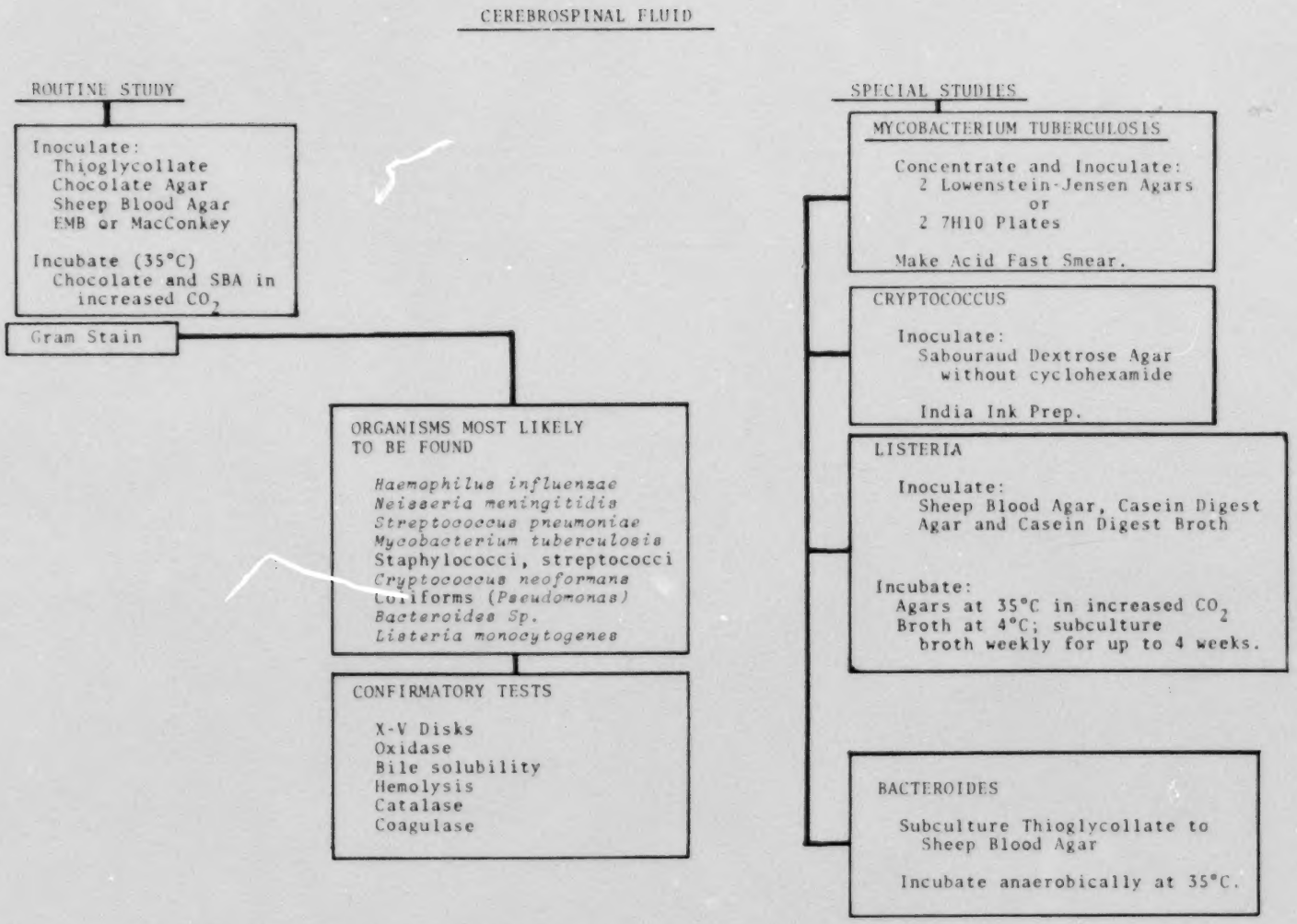
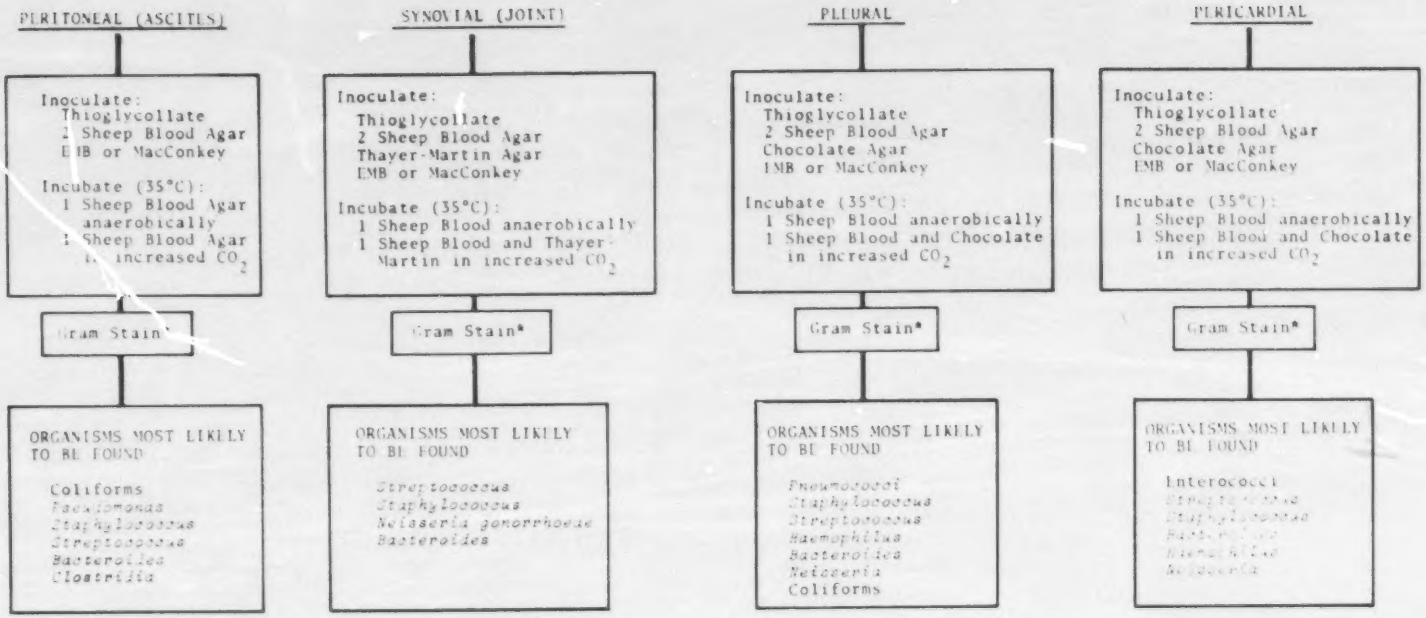


Figure 2–9. Technique for Cerebrospinal Fluid (Synovical, Pericardial, and Ascitic).

BODY FLUIDS **



*NOTE: Perform gram stain immediately. Results will frequently aid in media selection.

**Anaerobic cultures: consult chapter 16 of this manual.

Figure 2-10. Technique for Body Fluids.

phate buffer with an equal volume of glycerol and refrigerate at 4°C to 6°C.

(2) Certain pathogens found in stools, particularly Shigellae, die rapidly on exposure, and in such suspected cases the specimen should be processed immediately when possible.

(3) If a hard-formed stool is passed with mucus, pus, and bloody flecks on and in the stool, this is the specimen of choice.

(4) Stool specimens are always collected by the patient. Specific instructions must be given to assure that the patient does not take the specimen from a commode or bedpan after passage. The specimen should be taken directly as it passes out of the anus.

(5) A flowchart for processing stool specimens is shown in figure 2–11.

i. **Surgical Tissue and Autopsy Material:**

(1) Solid fibrous tissue must be minced or ground in a tissue grinder prior to being processed. Tissue can be minced by placing it in a sterile glass culture dish and cutting it into small pieces with scissors or a scalpel. If a tissue grinder is used, a small amount of sterile saline should be added to the specimen.

(2) Anaerobic cultures, if done, require that the specimen be processed immediately.

(3) Autopsy and tissue specimens can be of great danger to the person handling them. They should be processed with care.

j. **Gastric Washings:**

(1) Gastric washings are removed from the patient by means of a tube passed down the esophagus. Specimens are taken primarily by the physician or nurse.

(2) Gastric specimens are taken primarily to diagnose tuberculosis. Tubercle bacilli which are coughed up by the patient and swallowed are found collected in the stomach. Specimens are taken in the morning as soon as the patient awakens, and immediately neutralized using 10 percent sodium carbonate.

(3) Specimens are usually taken in a series of three.

(4) Specimens not processed immediately should be refrigerated.

(5) Specimens are sometimes taken in certain stomach cancer conditions. These specimens are taken at any time, and a smear is prepared to check for Boas-Oppler bacilli and Sarcina organisms.

k. **Gum Scrapings.** Scrapings of the gums are taken in diagnosing Vincent's angina, a disease producing a thick, purulent covering on the gums. The specimen is taken using a tongue depressor or similar sharp-edged instrument, and a smear is made and stained using a crystal violet stain. The disease is diagnosed by finding fusiform and spiral organisms together on the smear in large numbers. This spiral organism is one of the few spirals that can be seen after staining.

2–4. Specimen Shipment. Occasionally specimens are transported from one facility (smaller) to a reference (larger) laboratory for further analysis. Common methods for transporting specimens between laboratories are local carriers and regular mail. Depending on the mode of transport, certain points should be considered. If the reference laboratory is local, delivery of specimen is simple, requiring only the minimum amount of packing and protection against hazard. However, in instances in which the mail is the most prudent means of transport, regulations on mailing biological samples should be consulted. Specimens should be considered as to the period of time in the mail and viability of organisms upon reaching the destination. Whenever possible, obtain a laboratory manual or brochure from the facility being consulted for specific instructions on sample submission procedures. This can save much time and prevent unnecessary delay for any reason. Mailing of bacteriological samples involves consideration of the specimen, the media used, the containers, and the postal regulations.

a. **Specimens mailed** to distant facilities vary widely in type; they may be serum, urine, body fluid, fecal sample, and occasionally a viral study. Bacterial specimens can be noninfectious or infectious, aerobic or anaerobic. Each sample requires slightly different handling to insure reaching the destination in good condition.

(1) *Noninfectious* specimens are placed in sterile glass containers with a screw cap. Place the container in a standard double shipping container. Pad the spaces between the containers to guard against breakage using cotton or soft packing materials.

(2) *Infectious* specimens are placed in sterile stout glass tubes and sealed at the end by fusion of the glass. Place the sealed tube in a stout insulated screw cap or rubber stoppered tube, sealed with paraffin. Add 4 percent formaldehyde to the outer tube so that the liquid surrounds the inner sealed container, and seal by replacing the cap. Pack this double glass container in a standard double shipping container. Pad the spaces between the containers to guard against breakage. A cross section of a

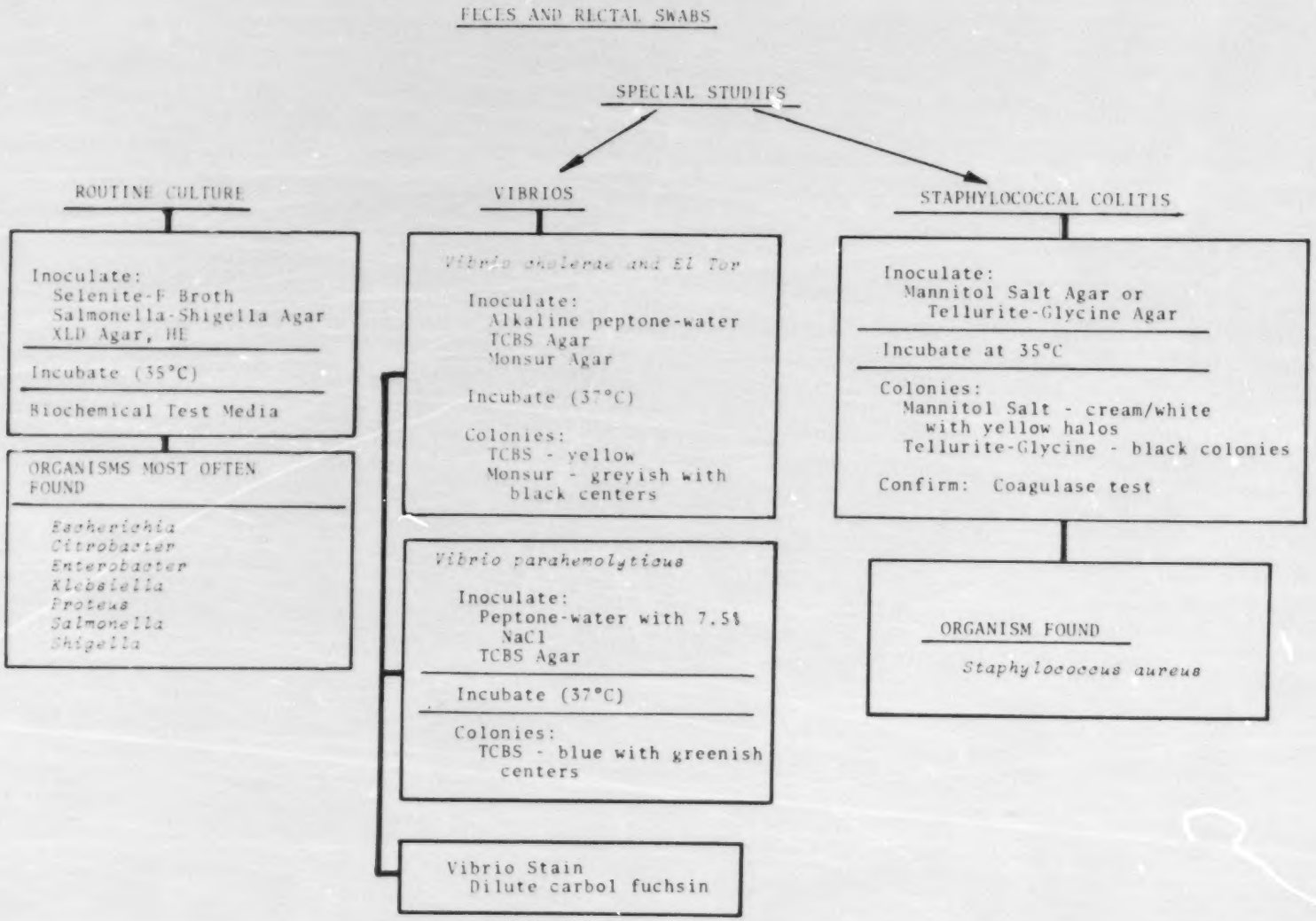


Figure 2–11. Technique for Feces and Rectal Swabs.

properly packed container is shown in figure 2–12.

(3) Place *aerobic* specimens in sterile glass screw cap tubes containing either broth or an agar slant, and place the tubes in the standard double shipping container. Specimens also may be sealed in the original petri dish or they may be subcultured to a second plate and sealed around the edge with paraffin strips to prevent accidental contamination or spillage during transport. The samples are then wrapped in a small amount of packing materials and placed in a sturdy shipping box for added protection and secured with several pieces of tape.

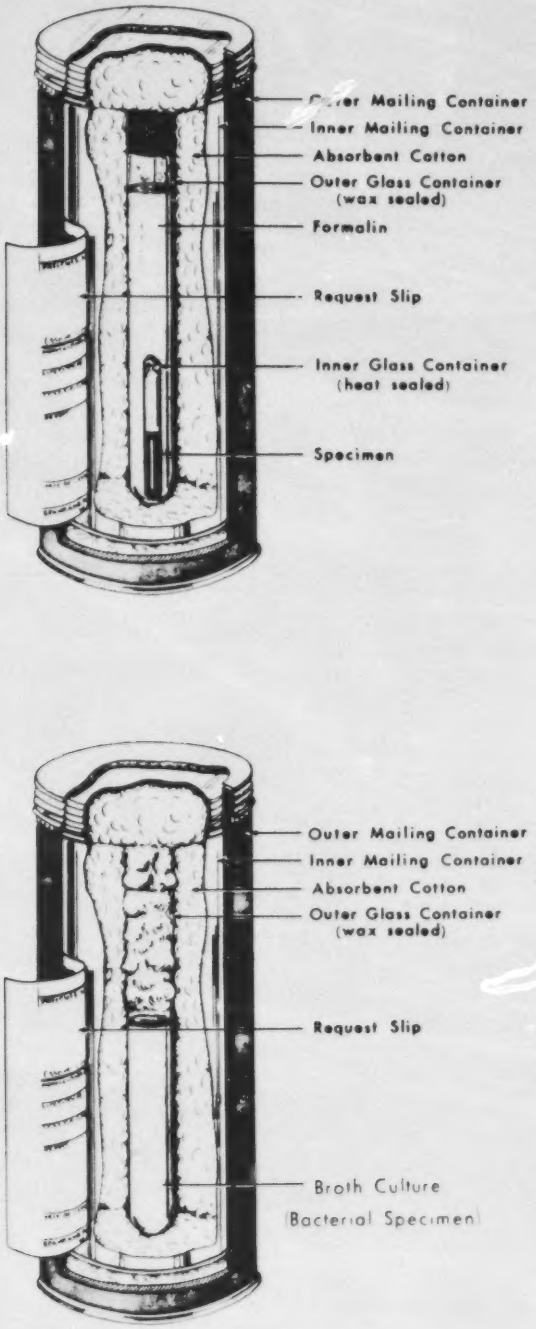


Figure 2–12. Cross-Section of Shipping Containers.

(4) Prepare *anaerobic* specimens as deep agar cultures in glass tubes or ampules, and seal by melting the open end over a burner (fusion). Care should be taken not to overheat the tube while sealing it or the media will rise to the top and scorch, causing damage to the specimen. The amount of agar in the tubes should not allow a large airspace at the top of the tube, minimizing aeration of the culture in transit.

b. **Media.** Media used in the shipment of specimens should support bacterial growth up to 72 hours in the mail. There are three transport media available from Difco: Stuart's Transport, Amies Transport, and SP Hajna's Formula. Stuart's Transport is an excellent media for the successful transport of *Neisseria gonorrhoeae, Haemophilus influenza, B. pertussis, Streptococci, Staphylococci, Pneumococci, Shigella, Salmonella, Escherichia,* and *Klebsiella* species. Amies is a modification of Stuart's medium (Cary and Blair) and is excellent for the transport of microbial specimens by mail. The medium is modified to yield a significantly high percentage of positive cultures as compared to the use of Stuart's original transport media. SP Hajna is a preservative for clinical specimens recommended for the transport of stool specimens or rectal swabs for the isolation of members of the *Salmonella* and *Shigella* groups and other Gram-negative bacteria.

c. **Containers.** Containers used for the shipment of specimens will vary dependent upon the number and size of specimens being shipped. However, as a general rule, the containers should be constructed of a strong, durable material capable of holding moisture within, in the event of breakage, and provide protection for the contents from outward damage in handling during transit. This material should not be heavy or too bulky as this would increase mailing cost unnecessarily.

d. **Postal Regulations.** Postal regulations require that packages sent through the mail must

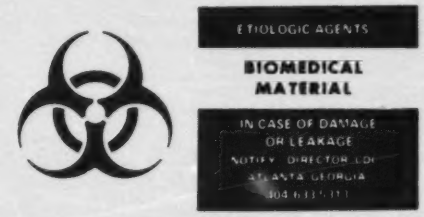
be properly prepared to prevent leakage, breakage, or temperature damage during transit. Also, a label should be affixed spelling out the contents and warning personnel of any potential danger of contamination from contents if mishandled. AFM 160–28 contains information on packaging of pathological specimens for the mail and should be consulted when large packages are prepared for mailing. Packages containing informational letters concerning the specimen must have first class postage affixed for shipment. Mailing containers should have affixed on the outside a label which reads: "Biomedical Material, Etiologic Agents. In case of damage or leakage notify: Director, CDC, Atlanta, Georgia, 404/633–5313." The labels (available from the *Federal Supply Catalog* under the stock number FSN 7690–00–082–9705) are shown in figure 2–13.

2–5. Collection and Shipment of Viral Specimens. The number of specimens that may be collected on any given patient is limited. These specimens, as a general rule, should be obtained from the anatomic area considered to be involved in the pathologic picture. For the exceptions, one must rely upon experience. Polio viruses, for example, are more readily isolated from stool samples than from central nervous system sources. A general schema for specimens to be considered is shown in figure 2–14. It indicates the types of patients presenting themselves and the various specimens that may be obtained. Obviously, there are always patients with symptoms that do not follow the usual patterns. Such patients may be thought of as simply "sick." Laboratory specimens in these cases generally consist of serum collected from the patient or "anatomical specimens."

a. **Serologic Specimens:**

(1) Serums obtained early in the course of disease (acute) and during convalescence (convalescent) are usually employed for serologic examination. These specimens must be obtained at optimal times so that antibodies will develop for possible identification (see figure 2–15.)

(2) Convalescent serums alone have little value. It is usually impossible to define a "diagnostic titer" or to interpret the significance of a titer in a single specimen. In apparent infection or a mild undiagnosed case at some undetermined time may have been responsible for the titer observed.



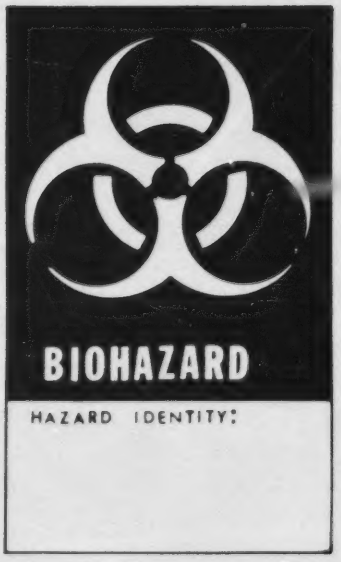


Figure 2–13. Warning Labels for Biologicals.

Patient*

Resp. Disease	CNS Disease	Exanthems	"Sick"**

Serology
Acute and Conv. Serums

Isolation

1. Throat washing (swab)***
2. Stool
3. Spinal fluid
4. Whole blood

* Autopsy or biopsy material—brain, lung, spinal cord, intestine, etc.

** No particular disease pattern.

*** Usually only specimen required for isolation of a respiratory agent.

Figure 2–14. General Schema of Specimen Collection for Viral Studies.

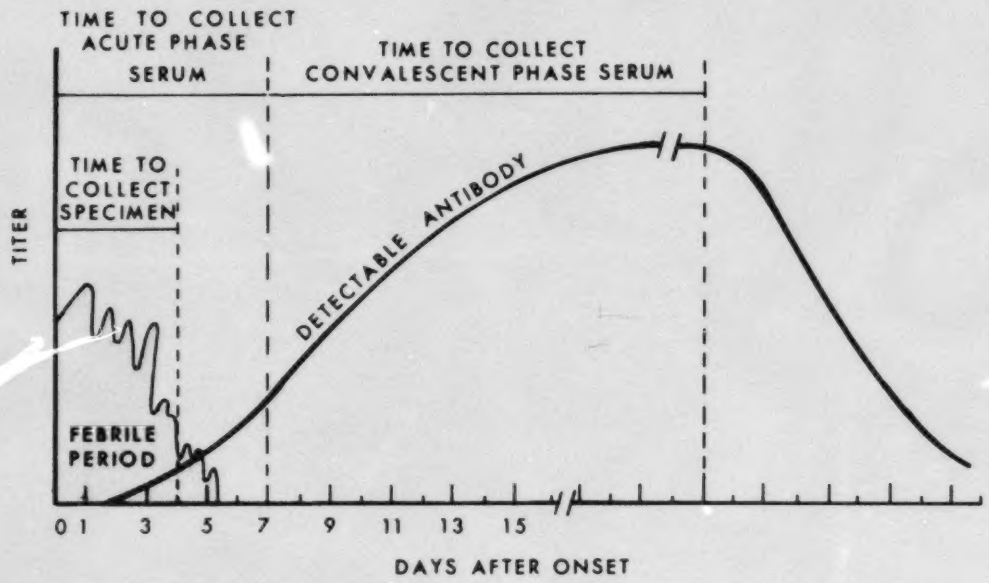

TIME TO COLLECT ACUTE PHASE SERUM
TIME TO COLLECT CONVALESCENT PHASE SERUM
TIME TO COLLECT SPECIMEN
TITER
DETECTABLE ANTIBODY
FEBRILE PERIOD
0 1 3 5 7 9 11 13 15
DAYS AFTER ONSET

Figure 2–15. Optimal Antibody Production Graph.

(3) Furthermore, what may be a high titer for one individual may be low for another. In certain tests, titers of 1:16 and 1:32 are "high." Thus a change from less than 1:4, 1:4, or even 1:8 to a value of 1:16 or 1:32 needs careful evaluation and may confuse the inexperienced. On the other hand, a high titer on a single serum sample may logically be used as suggestive evidence in certain instances. For example, a high serum CF titer to soluble (S) antigen early in cases of mumps associated with a low viral (V) antibody, along with clinical findings and other laboratory evidence commensurate with this illness, may be interpreted as highly suggestive of a current infection.

(4) Anamnestic reactions may also account for an elevated titer. For significance, a fourfold increase in titer must be demonstrated by one or another acceptable serologic procedure before considering an association of agent with disease. In general, an interval of 2 to 3 weeks between specimen collections is sufficient to detect this antibody change.

(5) As a rule, acute serums are collected as early as possible in the course of illness. It should be emphasized that it is impossible to go back in time and collect an acute serum after a few days of illness have elapsed. Make a routine practice of collecting admission or initial serums in all cases of suspected infectious illness, the diagnosis of which may conceivably require laboratory confirmation.

(6) Laboratory experience is important in determining which procedures will best suit the serums on hand. Table 2–2 indicates the different optimal times for detecting antibody by CF (compliment fixation) and by Nt (neutralization) and HI (Hemagglutination-Inhibition) tests.

(7) It cannot be sufficiently emphasized that a laboratory diagnosis can be made only upon the demonstration of fourfold (or higher) rise in antibodies when acute and convalescent serums are simultaneously tested. For immediate testing, serum specimens may

Table 2–2. Representative Viral Diseases and Observed Serological Response According to Time of Collection and Serological Test Performed.

Virus disease	*Serologic test*	*Antibody peak in days*
Influenza	CF	7–10
	HI	19–21
Mumps	CF(S)	5–10
	CF(V)	10–21
Poliomyelitis	CF	6–30
	MI*	(Onset of clinical disease)
Lymphocytic choriomeningitis	CF(S)	21 (approx.)
Western encephalitis	CF	21–45
	Nt	5–7
St. Louis encephalitis	CF	5–7
	Nt	21–45

*Metabolic Inhibition test.

be stored at 4°C. If longer storage is necessary, it may be advisable to freeze the serum samples, thus diminishing the possibility of growth of a contaminant and of the development of anticomplementary activity. The relative merits of serum storage have not been adequately studied, and there are differences in opinion pertaining to the most appropriate method of maintaining serums prior to testing.

b. **Anatomical Specimens:**

(1) The specimen of choice is obviously that which will most likely incorporate the agent in question. For example, throat washings (or swabs) or lung specimens (in fatal cases) are indicated when respiratory disease is in question; spinal fluid or brain (in fatal cases) is the specimen of choice when central nervous disease is under consideration. Experience is necessary at times in the isolation of agents that infect one area primarily and then give rise to symptoms by secondary invasion of another area, as frequently seen in enterovirus infections.

(2) Generally, the most advantageous time to collect specimens for virus isolation is before the actual symptoms are observed. This, obviously, is of little practical value. For best results, the optimal time of collection is during the febrile period or as early in the course of the disease as possible (refer again to figure 2-1).

(3) As always, exceptions are frequently encountered. Polio viruses are, for example, often excreted months after onset of illness. A summary of clinical disease, together with the most appropriate specimens to be collected, is given in table 2-3.

c. **Shipment of Specimens:**

(1) Viruses vary in their stability under deleterious conditions. Inasmuch as specimens for isolation studies contain agents of unknown characteristics, they must be handled in a manner capable of protecting any viruses present. Until more information is available, the recommended method for preservation of a virus is freezing of the specimen, best accomplished by placing the material in a suitable sealed glass container and submerging in a dry ice and alcohol bath. If the specimen is liquid, rotation (shell-freezing) speeds the process. Unavailability of dry ice may necessitate substitute methods of freezing, such as placing specimens into any available freezing compartment (see figure 2-16).

(2) For short periods of time, specimens may be transported or held on wet ice at approximately 4°C. Since stability of the agent is an unknown factor, refrigeration is only a temporary measure. Glycerine (15 to 50 percent in buffered saline) may be used for solid specimens but is of little value in preservation of liquids. When there is doubt, the specimen should be brought directly to the laboratory, where adequate handling by the most expeditious means is available.

(3) Serums for serological examination need not be shipped frozen. They may be shipped to the diagnostic laboratory by regular mail, but airmail is preferable. When a blood specimen is taken for isolation and serologic studies, it should be handled like any material submitted for isolation work. A small portion of whole blood is separated from the sample and frozen. The remaining portion is allowed to clot and the serum removed after centrifugation. This serum specimen is preferably kept frozen until the convalescent serum is taken in 2 to 3 weeks.

(4) Time and specimen quantity are often saved by coordinating collection and handling of specimens with the diagnostic laboratory involved.

(5) A valuable specimen is often lost by failure to place and ship it in a suitable container. Liquid specimens must be prevented from leaking. Although solid specimens do not leak, care must be exerted to prevent inactivation by improper handling, CO_2 fumes, breakage, etc.

(6) Leakage may best be prevented by adequately sealing the container. Since air express constitutes a major means of transportation, *changes in atmospheric pressure must be anticipated.* Screw-capped tubes may appear to be satisfactory but will often develop leakage. Rubber gaskets will help, especially when the seal is further strengthened by adhesive tape. Glass-sealed ampules are most satisfactory but may be too troublesome for routine purposes. Practically, rubber-stoppered tubes usually make a satisfactory seal which, reinforced with adhesive tape, prevents "popping" of the stoppers due to changes in pressure (see figure 2-16).

(7) Shipping specimens with dry ice (solid CO_2) entails the following precautions:

(a) Sufficient CO_2 to last the contemplated trip.

(b) Adequate insulation of package to prevent rapid evaporation of CO_2.

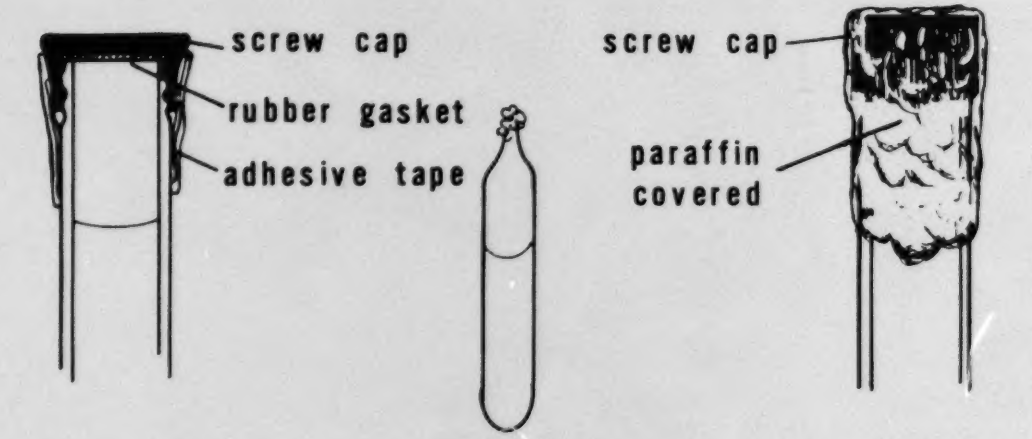
(c) Prevention of breakage of specimens owing to increased space in container as CO_2 evaporates.

Table 2–3. Clinical Disease and Appropriate Time of Collection.

Clinical disease	*Specimens for isolation*	*Time to collect serum samples*	
		Acute	*Convalescent*
Respiratory disease			
Influenza	Throat washing (swab)	Immed. after onset	2-4 wk. later
Para-influenza	Throat washing (swab)	Immed. after onset	2-4 wk. later
Adenovirus	Throat washing (swab)	Immed. after onset	2-4 wk. later
Primary atypical pneumonia	Throat washing (swab)	Immed. after onset	2-4 wk. later
Psittacosis	Throat washing (swab) (sputum)	Immed.	2-4 wk. later
Central nervous system disease			
Eastern encephalitis	Brain; blood	Immed. after onset	2-4 wk. later
Western encephalitis	Brain; blood	Immed. after onset	2-4 wk. later
St. Louis encephalitis	Brain; blood	Immed. after onset	2-4 wk. later
Herpes simplex	Vesicle fluid; blood		2-4 wk. later
Mumps	Blood; saliva	Immed. after onset	2-4 wk. later
Lymphocytic choriomeningitis	Blood; brain; spinal fluid	Immed. after onset	2-4 wk. later and 8 wk. later
Rabies	Brain	Immed. after onset	2-4 wk. later
Coxsackie	Stool; spinal fluid; throat washing	Immed. after onset	
ECHO	Stool; throat washing		
Polio	Stool; throat washing		
"Aseptic meningitis"	Stool; throat washing		
Exanthems			
Variola-vaccinia	Vesicle fluid; scrapings	Immed. after onset	
Chickenpox-herpes zoster	Vesicle fluid; scrapings		2-4 wk. later
Measles	Vesicle fluid scrapings; blood		
ECHO	Stool		
Rickettsial diseases			
Spotted fever group	Blood	Immed. after onset	
Typhus group	Blood		
Q fever	Blood		
Miscellaneous			
Psittacosis-lymphogranuloma venereum group; (Trachoma and Inclusion Conjunctivitis)	Sputum; plus smears	Immed. after onset	2-4 wk. later
Cat Scratch	Pus		2-4 wk. later
Epidemic Keratoconjunctivitis (See Adenovirus)			
Salivary Gland Virus (cytomegalic inclusion body)	Throat swabs; urine		2-4 wk. later

SHIPPING SPECIMENS

(Allow for changes in atmospheric pressure)



HANDLING SPECIMENS

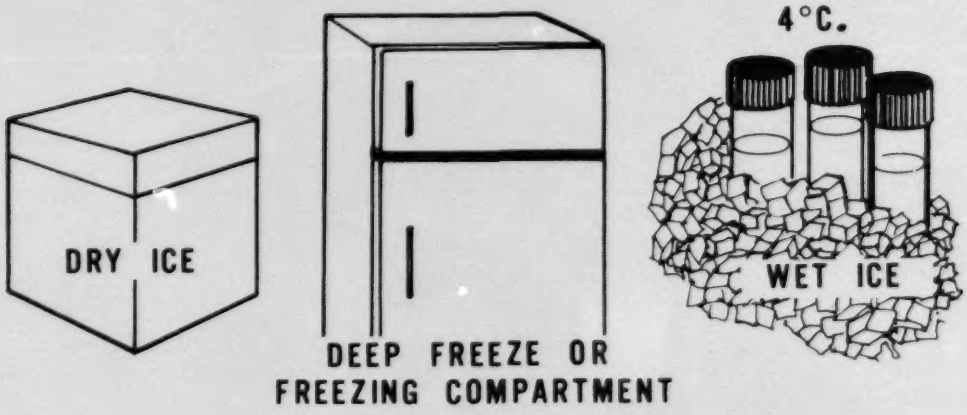


Figure 2–16. Handling of Specimens Prior to Shipping/Several Methods of Sealing Liquid Specimens Prior to Shipping.

(d) Protection of specimens from CO_2 fumes.

(8) Inexpensive apparatus may be obtained for sealing specimens in tin cans. Such containers with adequate packing material provide excellent protection for shipping.

(9) Specimens may be packed with wet ice by placing ice, together with the specimens, in a plastic bag, thus preventing leakage as the ice melts. Metal ice containers which prevent leakage of fluid are also available. These are kept frozen by storage in a deep freeze.

d. **Storage of Specimens.** Viruses vary in their stability to heat and cold, and generalizations are not desirable; however, the following recommendations may be considered:

(1) Viruses should be stored at the coldest temperatures available. In general, household-type freezers will keep most viruses for long periods of time.

(2) Refrigeration at −70°C (electric) is best for long-term storage of stock strains. Precautions must be taken to prevent CO_2 leakage into vials of virus. Glass-sealed ampules or rubber-stoppered tubes taped with adhesive may be used (refer again to figure 2–16).

(3) Multiple freezing and thawing of specimens are to be avoided.

(4) Lyophilization is an excellent method for preserving many of the viruses. Lyophilized agents may be kept at 4°C.

(5) Ordinary refrigeration temperatures (4°C) maintain many viruses for several days.

(6) The suspending medium will markedly influence the stability of the specimen. A buffered, neutral pH is usually preferred for long-term storage.

e. **Information Accompanying Specimens:**

(1) The potential number of tests necessary to arrive at a satisfactory diagnosis may be so numerous as to incur considerable expense. It is necessary for the physician, therefore, to make representative clinical data available to the diagnostic laboratory. The latter will assist laboratory personnel in deciding which procedures are most appropriate for testing the specimens under consideration, since the various tests cannot be done simultaneously.

(2) This information need not be extensive. Figure 2–17 is a sample diagnostic request, designed to include the following pertinent information: age, date of onset of illness, vaccinations, dates of collections of specimens, main clinical findings, and provisional diagnosis.

Requested by: _____ Lab. No. _____

_____ (Location) Date Received _____

Patient's Name _____

Address _____

_____ (State)

Age _____ Race _____ Sex _____ Occupation _____

Vaccination against viral or rickettsial agents in past:

3 mos. 9 mos. 12 mos. or year: 19 ____

Vaccine: Influenza Smallpox Yellow fever Polio

Typhus Spotted Fever Other _____

Date of onset of illness _____ Date specimen sent _____

Specimen and date collected: Whole blood _____, _____, _____

Serum, acute _____ Stool _____

convalescent _____ Throat washing _____

Spinal fluid _____, _____, _____

Tissue (specify) _____, _____, _____

Clinical data: Febrile Afebrile Rash: Yes No

Respiratory tract involvement: Yes No

Gastrointestinal tract involvement: Yes No

Meningitis: Yes No Paralysis: Yes No

Bulbar: Yes No Fatal: _____

Spinal fluid cell count _____

Contact: Family Community Animal or birds Unknown

Therapy, if any: _____

Provisional clinical diagnosis: _____

Tests required: _____

Figure 2–17. Sample Requests for Viral and Rickettsial Diagnosis Service.

Chapter 3

PREPARATION AND STAINING OF BACTERIA FOR EXAMINATION

3-1. Introduction. The observation of bacteria under microscopic conditions is fundamental to the science of bacteriology. In clinical bacteriology, characteristics such as shape, cell groupings/arrangements, and staining qualities are of value in identifying the organism. One of the most distinctive characteristics of bacteria, which aids in their identification, is their selectivity in staining with certain dye solutions. Due to their small size and transparency, bacteria are difficult to see microscopically, especially if mucus, tissue or other substances are present. A practical way of solving this problem is to stain bacteria so that they will stand out prominently. The type of dye used may be a general stain that colors all bacteria or it may be a selective stain that colors bacteria of a given group a specific color and those of a different group a contrasting color. In this chapter the preparation of smears for staining, staining procedures, and microscopic examination of mounts (wet or unstained) will be discussed. Methods for staining special cell structures, such as capsules, flagella, and spores, are also included in this chapter.

3-2. Mechanics of Staining. Why dyes will stain one material or organism and not another is a subject not yet fully understood. It is known, however, that a staining reaction is due to physical as well as chemical factors.

a. **Physical Factors:**

(1) Substances that stain must be somewhat porous. Penetration of dyes into these substances can be a result of capillary and osmotic forces.

(2) Absorption occurs in some staining processes: molecules of the dye are able to selectively penetrate the molecular spaces of a substance and to remain within it in a solid state solution.

(3) Adsorption is the mechanism in some other processes: the dye is able to adhere (accumulate) in high concentration on the surface of the dyed substance.

b. **Chemical Factors:**

(1) Bacterial cells, proteins, mucus, and tissue, contain components, some of which are acidic and some are basic. This characteristic allows chemical reaction to occur between cations or anions of a dye and those of the component, thus dyeing the substance.

(2) Protein, the primary constituent of living cells, is composed of helical chains of amino acids and is amphoteric in nature. That is, protein contains atom groups that react as either cations or as anions, depending on the hydrogen ion concentration of the medium in which the protein is suspended. For example, at a specified pH known as the isoelectric point (pI) a protein molecule is electrically neutral, and the protein ion is said to be *zwitterion*. Such a protein ion will react as if it were an acid at a pH below its pI and as a base at a pH above its pI. Thus, protein ions are able to enter into true chemical reactions with other electrolytic substances, such as acids, bases, and, in this case, dye salts.

3-3. Types of Microbiological Stains:

a. **Simple Stains.** A simple stain is one which indiscriminately stains all of the sample the same color. It is used in determining general shape and size of organisms and in counting them. Occasionally, a simple stain is used negatively. In a negative staining procedure the background material is stained, leaving the organisms unstained and distinguishable. The simple stains that are commonly used are listed below. Preparation of these and other stains are given in attachment 1.

(1) Buffered methylene blue (Loeffler's).

(2) Nigrosin (India ink).

(3) Dilute carbol-fuchsin.

b. **Differential (Selective) Stains.** A differential or selective stain is one that discriminates organisms on the basis of specific colors or cellular reactions that result from the staining process. This is the most important and useful type of stain. In many instances, very accurate tentative diagnoses can be made based on differential staining. For example, gonorrhea, a venereal disease, is commonly detected in males by using a stained smear. Also, results of differential staining in many instances provide the basis for determining what specific type of culture and test will be necessary to identify a pathogen. The most important differential stains are:

(1) *Gram Stain.* This stain was devised by Hans Christian Gram in 1884. This stain is the single most useful and important stain used in the microbiology laboratory. In principle, a crystal violet-iodine complex is applied to a heart-fixed and cooled smear. Certain orga-

nisms become stained so strongly that they resist decolorization by an alcoholic solvent. Other organisms apparently only slightly absorb the stain, and the stain can be easily removed by the alcoholic decolorizer. A counterstain is applied to the smear to give the nonstaining organism or background material a distinctive opposing color. Bacteria, as a result of the above process, are divided into two groups. Those that adsorb the crystal violet-iodine complex and resist subsequent decolorization are referred to as *Gram-positive* and stain purple. Organisms that do not retain the crystal violet, and therefore become decolorized, are referred to as *Gram-negative*. The complete process that takes place when an organism stains or does not stain is not fully known. It is thought that organisms stain Gram-positive because their cell wall proteins have a greater affinity for the crystal violet-iodine complex and that this allows them to enter into true histochemical reaction with the dye. Studies have shown that in Gram-positive cell walls there is a greater concentration of glycopeptides than in Gram-negative cell walls, while Gram-negative cell walls have a higher concentration of lipids than Gram-positives; this may account for the dye-iodine-protein interreaction. Other studies suggest that staining may be due to some form of denaturation of the cell-wall protein by the alcoholic solvent and this denaturation decreases cell wall permeability of Gram-positive cells, trapping the dye-iodine complex within the cell.

(2) *Acidfast Stain (Ziehl-Neelsen)*. The acid-fast stain is used to differentiate *Mycobacterium* and *Nocardia* species from other organisms. The stain is very important because it is used to detect tuberculosis, a serious and often fatal illness. Basic fuchsin, a compound dye consisting of pararosanilin, rosanilin, and magenta II, is applied in a solution made acid with phenol (carbolic acid) to a fixed smear, and the smear is then heated. A hydrochloric acid-alcohol decolorizer is applied, and acid-fast organisms resist decolorization, retaining the dye and remaining red in color. Organisms which are not acid-fast are decolorized and are colored by a counterstain (usually methylene blue is used). Microscopic examination of a smear containing acid-fast organisms will show distinct red, rod-like, acid-fast bacteria against an evenly blue background. Reaction of the bacterial cell-dye complex is thought to be due to the high lipid content of acid-fast cell walls. The carbol fuchsin dye appears to be more soluble in the cell wall lipids than in the acid-alcohol decolorizer, and therefore acid-fast cells retain the red dye. Damage to the cell wall has been shown to result in a loss of acid-fastness.

(3) *Capsule Stain*. Capsule stains are in effect negative stains. That is, organisms possessing a capsule are themselves stained, but the capsule is not. Microscopically stained preparations usually show a tinted organism surrounded by a clear unstained capsule. Background material is also tinted, and this allows distinct differentiation of a bacterium and its capsule. Basic dyes are used because they have an affinity for cell nucleic acids but not for capsular material.

(4) *Spore Stains*. Bacterial spores are resistant structures containing high concentrations of nuclear material. This nuclear material is composed primarily of nucleic acids and therefore has a greater affinity for basic dyes than other cellular material. Smears generally show small round or oval spores stained a distinctive color against a contrasting counterstained background. Spore stains are primarily used in differentiation of Genus *Clostridia*.

(5) *Flagella Stains*. Flagella are too fine to be seen with the ordinary compound microscope. They are made visible on smears by coating flagellated organisms with a dye complex which builds up in layers. Flagella coated in this manner appear larger than they actually are and therefore are easier to see. The dye will coat both the main cell body and flagella an indiscriminate color.

(6) *Granule Stains. Corynebacterium* bacilli and a few other species possess internal structures called metachromatic granules. These structures contain phospholipids, nucleic acids, and a small amount of protein. They will stain with most basic dyes, such as basic fuchsin, crystal violet, and methylene blue. Because the affinity for the dye is greater for the granules than for other portions of the cell, a stained bacterium will exhibit a banded appearance of contrastingly darkly stained granules alternating with lighter stained cell cytoplasm. Metachromatic granule stains are used primarily to differentiate and identify *Corynebacterium diphtheriae*, which causes serious disease in children.

3–4. Preparation of Smears for Staining. Bacterial specimens for staining are prepared on glass microscope slides. Slides should be checked for the presence of any debris which could interfere with the examination of the finished product. Debris may include dust, dirt

particles, wax, fingerprints, lint, or cotton fibrils. Any of these may appear as an artifact and be mistaken for a significant find in the diagnosis of a disease by an inexperienced technician.

a. **Bacterial Specimens.** Bacterial specimens are those samples suspected of containing pathogens which are submitted for examination. Slide preparation of specimens should be as follows:

(1) *General Considerations:*

(a) In preparing slides for examination, multiple smears increase the possibility of observing etiological agents.

(b) If the specimen is to be cultured, care must be taken not to contaminate it when other tests are to be performed on the same sample. It is best to plant the cultures first and then make smears for staining. Some of the uncontaminated specimen should be saved in case unsuspected organisms are observed and special media are required for their isolation.

(2) *Exudates/Pus.* Place material on a clean slide with a sterile wire loop. Care should be taken to prepare a thin smear of material. Avoid making smears too thick for microscopic examination.

(3) *Swabs.* Clinical samples submitted in swabs for examination should be smeared for staining, if requested, immediately upon receipt. This is the best manner to handle this type of sample as it would be less practical to remove the swab from a culture media to prepare a slide. However, in the case of drying swabs, a small amount of sterile broth is helpful in salvaging a specimen.

(4) *Sputum.* Care should be taken when preparing these specimens for analysis; select any flecks of mucus or blood-tinged particles present, since they are more likely to show infectious agents on microscopic examination. Do not make the smears thick or the morphological features of the bacteria will be difficult to determine.

(5) *Fluid Specimens.* Preparation of slides from specimens such as urine, cerebrospinal fluid (CSF), and aspirated samples (synovial, pleural, or peritoneal fluids) may require centrifugation to concentrate any bacteria present. After the specimen has been centrifuged, the supernatant fluid should be poured off into another container until it can be autoclaved and discarded. The concentrated portion can be placed on a slide and air-dried prior to heat-fixing. An applicator stick can be used to transfer and spread the material onto the slide. For those specimens containing little protein (urine), it may be helpful to add egg albumin (sterile) to insure that the material adheres to the slide and does not wash off during the staining procedure.

(6) *Tissue Specimens.* Tissue specimens suspected of harboring bacteria can be cut into small sections if necessary to increase the surface area and smears prepared for staining. A sterile cutting device (surgical blade, razor blade, or knife) should be used to prevent the possibility of introducing a contaminant into the specimen. A glass slide (sterile) should be pressed against the surface of the slide. The preparation is then allowed to dry at room temperature and then passed over an open flame several times to heat-fix the smear. A portion of the tissue should be saved for culture since smears prepared in this manner are not always positive.

b. **Specimens From Broth Cultures.** When fluid cultures are examined for growth, prepare smears by taking a loopful of the medium and spreading it on a glass slide. If the broth is highly turbid, spread the droplet over an area about the size of a dime. When growth is scant, it may be necessary to centrifuge the broth or use several loopfuls of medium to concentrate the organism on the slide.

c. **Specimens From Solid Media.** If smears are to be prepared from a culture on solid media, pick a minute amount of growth from a pure colony and emulsify it in a droplet of sterile saline (or sterile distilled water) on a glass slide. This is best accomplished using a straight inoculating needle or loop. The suspension of organisms should be only slightly cloudy; otherwise the smear will be too thick for microscopic observation of individual cells and accurate morphological interpretation.

3–5. Method for Preparing Smears. The steps in preparing a smear are: selecting a clean slide, preparing a thin-film smear to dry, and finally heat-fixing the smear. Routinely, it is a safe practice to prepare all smears in a bacteriological safety hood.

a. **Selecting Clean Slides.** The first step in making a smear for staining and microscopic examination is to select clean slides. Use new slides whenever possible; also, cleaning them as follows prior to use is suggested:

(1) Clean all slides to be used in a chromic acid solution.

(2) Rinse well in water two or three times, and place the slides in 70 to 95 percent alcohol.

(3) Remove the slides one at a time from

the alcohol and dry immediately, using a clean, soft, lint-free cloth.

(4) Store cleaned slides in a box to prevent dust contamination.

b. **Preparing a Thin-Film Smear.** The specimen must be spread on the slide in a thin-film smear. This allows even staining and better separation of organisms. Thick films should be avoided, due to the fact that these thicker smears are very difficult to examine. If a specimen contains only a few organisms, centrifuge it to concentrate it before making the smear. Smears can be prepared as follows:

(1) From a tube culture:

(a) Place a small drop of distilled water on a clean slide.

(b) Flame the inoculating loop or needle to allow it to air-cool.

(c) While holding the inoculating loop, remove the plug or screw-cap from the tube with the other fingers of the same hand that is holding the loop. Do not lay the plug or cap down. Flame the mouth of the tube.

(d) Insert the loop into the tube and remove the specimen.

(e) Reflame the tube mouth and immediately replace the plug.

(f) Emulsify the specimen on the loop in the drop of water on the slide.

(g) Spread out into a thin film and air-dry.

(h) Several specimens can be put on a single slide if so desired. Divide a multi-smear slide into sections with a diamond point or wax pencil.

(2) From a culture dish:

(a) Flame the loop and cool.

(b) Turn the culture dish upside down so that the lid is on the table.

(c) Grasp the bottom of the petri dish with one hand, holding the sterilized loop with the other. Remove the bottom portion of the dish and hold it facing you obliquely. Try not to breathe directly on an open culture, to prevent possible contamination.

(d) Remove the specimen and replace the culture dish bottom into the top portion.

(e) Emulsify the specimen in a drop of water (sterile) and spread to a thin film on a glass slide. Air-dry.

(3) Direct smear:

(a) A direct smear is one prepared directly from a specimen from the patient.

(b) Select a suitable portion of the specimen to be used for culture. Bloody flecks, pus, and mucus, for example, should be selected over other specimens.

(c) If the specimen is tissue, it should be minced in a sterile glass petri dish using a small amount of water and a sterile scalpel. Select a portion of this specimen and smear it thinly on a clean slide. Air-dry.

c. **Fixing the Smear:**

(1) Using forceps, pass the smear back and forth through the flame of a burner.

(2) The flame should contact the bottom of the slide opposite the specimen.

(3) Hold the slide in the flame for 1 to 2 seconds on each passage.

(4) After each passage place the underside of the slide on the back of your hand, carefully, to check the temperature. The slide should gradually get warmer, not hot. A hot slide indicates overfixing. If the smear has not been properly air-dried, too much heat can pause spattering and distortion. Spattering can lead to your being infected if the organism is a virulent pathogen. Additionally, the slide may shatter at one of the etch marks.

(5) Allow the fixed smear to cool to room temperature.

d. **Disposing of the Smear.** After smears have been stained and examined, or if excess smears are made, dispose of them by immersing them in a good disinfecting solution (5 percent phenol solution). Do not allow smears to lie around longer than necessary, particularly if unstained.

3–6. General Rules for Preparing Staining Solutions. The many different stains incorporate many diverse steps in their preparation and use. A general method of preparing staining solutions applicable in all situations is not possible. However, observation of the following rules will aid you.

a. Use "certified" stains. These are stains which have been tested and found to be satisfactory. They are labeled as certified by the United States Commission of Standardization of Biological Stains.

b. When they are not in use, keep stain bottles tightly closed.

c. Weigh stains on an analytic balance (dry) during preparation.

d. Triturate (grind) dyes in a mortar with a small amount of the diluent. Allow to settle for a few minutes; then pour the liquid off into a stock bottle. Add more diluent and repeat trituration. Continue until all of the dye is dissolved; then add the balance of the diluent to the stock bottle.

e. Except for a few rare instances, freshly prepared stains should be allowed to stand for

24 hours before being used. The exceptions are generally in preparing simple stains.

f. Filter staining solutions before use. Most stains can be filtered and stored in a stock bottle after standing 24 hours. Others require filtering immediately before use. Whether filtered and stored, or filtered and used, filtration is a must in good staining results.

g. Do not use staining solutions containing precipitates. Filter or prepare a fresh solution.

h. Make diluted solutions of stocks in small amounts as needed. Stock solutions are more stable as a concentrate than in a more dilute form.

i. Label stain solutions and other reagents with a stain-proof label showing the name of the stain, contents, concentration, and any other information necessary.

j. Do not expose stain solutions to sunlight. Most stain solutions are adversely affected by continuous exposure. Store stock solutions in a dark place.

k. Use glass or a resistant plastic such as polypropylene to store staining solutions. Some stains will react with polyethylene and related plastics.

3–7. Staining Procedures:

a. **Acidfast Stain (Ziehl-Neelsen):**

(1) *Principle.* Members of the genus *Mycobacterium* and certain *Nocardia* spp possess relatively large amount of lipid content, including waxes, in their cell walls. These substances resist penetration and staining of the cytoplasm by ordinary methods. When these organisms are exposed to a high concentration of basic dye and heat is applied, staining is more readily accomplished. Once stained, the organisms will resist decolorization with 3 percent acid—97 percent alcohol even upon prolonged exposure. Although all bacteria may be stained in this manner, only the above organisms, with rare exceptions, will resist decolorization with the acid-alcohol solution.

(2) *Reagents:*

(a) Carbol-fuchsin (Ziehl-Neelsen) R-S (16).

(b) Acid-alcohol R-S (3).

(c) Löffler's methylene blue stain R-S (45).

(3) *Technique:*

(a) Place dry, heat-fixed slides on a staining rack and flood them with carbol-fuchsin stain.

(b) Heat slowly to steaming by passing a flame under the slides. Do not ignite the stain or allow it to boil or become dry. Heat intermittently 3 to 5 minutes.

(c) Cool and wash rapidly with tap water.

(d) Decolorize with acid-alcohol until no more stain (excess) comes off smear.

(e) Wash with tap water.

(f) Counterstain with methylene blue for 20 to 30 seconds.

(g) Wash with tap water, drain, and blot dry. Examine slide microscopically using an oil immersion objective.

(4) *Interpretation.* Organisms which retain the primary stain (carbol-fuchsin), after the smear is treated with acid-alcohol decolorizer, appear pink to red. They are, therefore, termed acidfast. All nonacidfast organisms are readily decolorized with acid-alcohol; consequently, they stain blue with the counterstain.

(5) *Discussion.* The Ziehl-Neelsen or Modified Kinyoun techniques are routinely used in examining the specimens or cultures for the presence of acidfast tubercle bacilli. The procedure used is determined by the preference of the individual worker. In the Ziehl-Neelsen technique, time and temperature of heating are somewhat critical. Overheating diminishes the color of the organisms to a pink or brown or may even distort the shape of the cell, causing false negatives to be reported.

b. **Acidfast Stain (Kinyoun Modified):**

(1) *Principle.* The Kinyoun technique is similar to the Ziehl-Neelsen technique except that a wetting agent, instead of heat, is used to facilitate staining.

(2) *Reagents:*

(a) Carbol-fuchsin (Kinyoun) R-S (15).

(b) Turgitol No. 7 (commercial item).

(c) Acid-alcohol R-S (3).

(d) Loffler's methylene blue stain R-S (45).

(3) *Technique:*

(a) Flood fixed smear with Kinyoun carbol-fuchsin.

(b) Stain for 2 minutes. Do not heat.

(c) Wash rapidly with tap water and drain.

(d) Decolorize with acid-alcohol to remove excess stain.

(e) Counterstain with methylene blue for 20 to 30 seconds.

(f) Wash with tap water, drain, and blot dry. Examine.

(4) *Interpretation.* Acidfast organisms will stain pink to red. Background and nonacidfast organisms stain blue.

(5) *Discussion.* Turgitol reduces the sur-

face tension between the cell wall of acidfast organisms and the carbol-fuchsin stain, as does heat in the Ziehl-Neelsen method. The heatless turgitol method is more advantageous in that it is less cumbersome and requires less time.

c. **Auromine O Stain-Blue Light (For Mycobacteria):**

(1) *Principle.* A heat-fixed smear is flooded with auramine O, a fluorochrome, and examined with ordinary incandescent light that has been filtered to deliver light at 300 to 400 nm (blue-violet). An excitation filter is placed between the light source and specimen to remove wave-lengths other than blue-violet. A barrier filter is used in the tube or ocular to transmit excited fluorescence.

(2) *Filters:*

(a) Excitation: Schott BG-12. No. 47 (two thicknesses).

(b) Barrier: Schott OG-1.

(3) *Reagents:*

(a) Auramine O Stain (Morse) R-S (8).

(b) Decolorizer (Acid-Alcohol) R-S (3).

(c) Counterstain (Potassium Permanganate) R-S (57).

(4) *Specimen Decontamination.* Treat the specimen with an equal volume of 1 percent sodium hypochlorite (20 percent solution of Colorox is satisfactory). Mix well. Centrifuge to concentrate. Prepare the smear and stain it immediately.

(5) *Procedure:*

(a) Flood heat-fixed and cooled smears with stain for 15 minutes.

(b) Wash gently in running tap water. Drain.

(c) Decolorize for 2 minutes.

(d) Wash in tap water. Drain and air-dry.

(e) Counterstain with $KMnO_4$ for 3 minutes.

(f) Coverslip and examine.

(6) *Interpretation:*

(a) Acidfast organisms show a yellow-orange fluorescence.

(b) Artifacts (nonacidfast bacteria and cellular debris) show a pale yellow fluorescence.

(7) *Discussion:*

(a) Do not allow more than 1 hour to elapse after decontamination before initiating staining. Sodium hypochlorite will cause disintegration of the organism on long exposure.

(b) Unless special fluorescent objectives are used, it is important that the coverslip is of a thickness that is compatible with the high-dry objective.

(c) The low-power objective can be used to scan the smear, switching to high-power for confirmation.

(d) A drop of nonfluorescing immersion oil or glycerol should be used as a mounting medium when the smear is coverslipped and the 400X high-power objective used.

(e) Fluorescent procedures do not identify acidfast organisms; they only detect their presence.

d. **Auramine-Rhodamine Stain-Ultraviolet (Truant et al):**

(1) *Principle.* Stained organisms are exposed to filtered ultraviolet radiation. Acidfast organisms fluoresce distinctively. This procedure is the recommended technique for fluorescent examination of sputum, gastric specimens, and other specimens.

(2) *Filters and Light Sources:*

(a) *Excitation:* Schott BG-12 or Corning 5113.

(b) *Barrier:* Schott OG-1.

(c) *Heat absorbing:* Two thicknesses required (of filters).

(d) *Lamp:* Mercury arc high intensity ultra-violet lamp.

(3) *Specimen Decontamination.* See paragraph 3–5c (4).

(4) *Reagents:*

(a) Auramine-Rhodamine Stain R-S (9).

(b) Decolorizer (Acid Alcohol) R-S (3).

(c) Counterstain (Potassium Permanganate) R-S (57).

(5) *Technique:*

(a) Fix smears by heating on a heat block at 65°C for 2 hours or overnight.

(b) Flood the smear with stain and let stand for 15 to 20 minutes at room temperature.

(c) Rinse gently in tap water.

(d) Decolorize in acid-alcohol.

(e) Rinse thoroughly but gently in running tap water.

(f) Counterstain with $KMnO_4$ for 2 to 5 minutes.

(g) Rinse with tap water, drain, and air-dry.

(h) Place a drop of low-fluorescing immersion oil or glycerol on the smear and coverslip.

(6) *Interpretation:*

(a) Acid-fast organisms fluoresce reddish, golden yellow against a dark background.

(b) Artifacts (cellular debris and non-acidfast organisms) fluoresce hazy yellow or grey-green and lack the reddish tinge.

(7) *Discussion:*

(a) Why the counterstain improves the smear is not fully known; however, results show that it helps the technique.

(b) The procedure can be done using either bright-field or dark-field condensers. The dark-field condenser gives a larger field of view and examination can be made at low powers without using oil between the top of the condenser and the slide.

(c) A microscope specially designed for fluorescent work is preferable to a jerry-built outfit because of the danger to the eyes from stray ultraviolet radiations.

(d) Coverslips must be of the right thickness to be compatible with the objectives on the microscope.

e. **Capsule Stain (India Ink):**

(1) *Principles* Capsular material is a thick, colorless, gluey substance found around some bacterial cells. It does not absorb India ink. Therefore, when capsulated bacteria are suspended in India ink, they will appear as clearly outlined colorless halos surrounding the dense centrally situated bacterial cell. This is a negative staining method.

(2) *Reagent:* India Ink (Drawing Ink) R-S (Commercial Source).

(3) *Technique:*

(a) Using a loop or inoculating needle, mix a small amount of bacterial growth in a drop of water on a clean slide.

(b) Add a small drop (loopful) of ink and mix well.

(c) Immediately add a coverslip, allowing the mixture to spread as a thin film beneath the coverslip. Do not use an excess of ink or specimen. The organisms should lie in a single layer beneath the coverslip.

(d) Examine immediately with oil immersion under reduced light.

(4) *Interpretation.* Capsules are seen as clear halos around dense bacterial bodies.

(5) *Discussion.* Not all brands of India ink are satisfactory for demonstrating the presence of capsules. Inks selected for use should be free of microbes. Suitable inks may be protected against contamination by adding sufficient phenol to bring the concentration to 0.5 percent, by volume. Those inks containing very finely divided carbon particles yield the best results. Capsules are best demonstrated in cultures 4- to 6-hours old.

f. **Capsule Stain (Hiss Method):**

(1) *Principle.* Bacterial capsules are composed of polysaccharide compounds which do not stain well by ordinary methods. Encapsulated bacteria exhibit capsular swelling when mixed with normal serum and is capable of being stained at that time, the basis of the Hiss method.

(2) *Reagents:*

(a) Crystal violet R-S (20).

(b) Copper sulfate R-S (17).

(c) Serum (normal human or rabbit).

(3) *Technique:*

(a) Mix a loopful of specimen, broth culture, or normal sterile serum on a glass slide. Spread the mixture. Allow the smear to air-dry.

(b) With the smear side up, heat-fix the preparation by passing it three or four times through a flame, taking care not to overheat it.

(c) Cover the smear with crystal violet stain and steam the preparation gently over a flame for 30 to 60 seconds.

(d) Wash off the stain gently with the copper sulfate solution, blot off the excess (gently), and allow the slide to dry at room temperature. Examine the slide using the oil immersion objective.

(4) *Interpretation.* With this method, the capsules appear as faint blue halos around dark blue to purple cells.

g. **Capsule Stain (Muir):**

(1) *Principle.* Capsules on bacteria are made visible by first coloring the bacterial bodies with a stain that is poorly absorbed by the capsular material. This procedure is followed by applying a counterstain that colors the capsular material a contrasting color.

(2) *Reagents:*

(a) Strong Carbol-Fuchsin R-S (13).

(b) Mordant R-S (49).

(c) Ethyl Alcohol 95 percent R-S (4).

(d) Methylene Blue R-S (45).

(3) *Technique:*

(a) Make a thin-film smear and air-dry. Do not heat-fix.

(b) Flood the smear with carbol-fuchsin and heat it gently as in the Ziehl-Neelsen technique for 1 minute.

(c) Wash rapidly but gently with 95 percent alcohol and then with water. Drain.

(d) Flood with mordant for 30 seconds.

(e) Wash with water, then with 95 percent alcohol until the smear is pink.

(f) Wash with water. Drain.

(g) Counterstain with methylene blue for 30 seconds.

(h) Wash, drain, and blot dry.

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(i) Examine with oil immersion.

(4) *Interpretation.* Bacteria stain red; capsules stain blue.

h. **Flagella Stain (Leifson Method Modified):**

(1) *Principle.* A flagella-staining solution contains a basic dye along with a substance composed of fine particulate matter in a colloidal suspension. Upon adding the stain to a dried smear of motile bacteria, the dye-covered particles are deposited on the flagellar filaments, rendering them microscopically visible.

(2) *Reagents:*

(a) Basic fuchsin-tannic acid staining solution R-S (10).

(b) Borax-methylene blue counter-stain R-S (11).

(3) *Technique:*

(a) Suspend a small amount of growth from agar medium in distilled water or normal saline to obtain a slightly turbid suspension. Place a large loopful of the suspension on a clean glass slide.

(b) Titlt the slide lengthwise and allow the suspension to run down to form a film. Air-dry the film; do not heat-fix.

(c) Flood the smear with the flagellar stain for 5 to 10 minutes; then wash, air-dry, and examine the slide using the oil immersion objective.

(4) *Interpretation.* The flagella of various bacteria appears as extremely slender, fragile filaments which may occasionally be spiraled.

i. **Gram Stain (Hucker Modified):**

(a) *Principle.* Bacteria are stained with crystal violet and an iodine mordant to set the stain. A decolorizer is applied, and it removes the stain from organisms that do not have a great affinity for the stain. A counterstain is applied, and it stains the decolored organisms. The procedure differentiates bacteria into two classes: (1) Those that retain the crystal violet stain are *Gram-positive*, and (2) those that do not retain the stain are *Gram-negative.*

(2) *Reagents:*

(a) Ammonium oxalate-crystal violet R-S (20).

(b) Gram's iodine solution R-S (23).

(c) Alcohol decolorizer R-S (1).

(d) Safranin, counterstain R-S (60).

(3) *Technique:*

(a) Prepare a thin-film smear on a clean slide and air-dry.

(b) Heat-fix.

(c) Flood the smear with the diluted crystal violet stain and allow to act for 1 minute.

(d) Wash the slide well in running tap water.

(e) Flood the slide with iodine mordant for 1 to 2 minutes.

(f) Wash it in running water.

(g) Decolorize with acetone-alcohol by flowing the decolorizer on drop by drop while holding the slide at one angle. Decolorization should continue only as long as excess stain is still coming off. When the drippings are clear, stop decolorization.

(h) Immediately wash the slide in running tap water.

(i) Flood with safranin counter-stain for at least 30 seconds but not more than 1 minute.

(j) Wash, drain, and blot dry.

(k) Examine with oil immersion objective.

(4) *Discussion:*

(a) Crystal violet used for staining gives best results if a certified grade reagent is used.

(b) It is important to dissolve the iodine in a small aliquot (5 ml) of water before diluting it further. Iodine does not dissolve readily if initially mixed with a large volume of water.

(c) Do not overdecolorize. The acetone-alcohol decolorizes rapidly. If desired, slower decolorization can be accomplished by using straight 95 percent alcohol.

(d) Wash in water carefully but thoroughly, because specimens are sometimes washed off the slide by a direct stream of water.

(e) Known Gram-positive and Gram-negative organisms should be run periodically as a check on the staining procedure in quality control.

(f) Do not prepare the iodine-mordant in extremely large volumes for working solution, because it fades slowly when allowed to stand and readily when exposed to light.

j. **Metachromatic Granule Stain (Albert Modified):**

(1) *Principle.* Several species of *Corynebacterium* contain within their cytoplasm dense granular dark-staining material called metachromatic or volutin granules, nonstaining bands, and normal staining cytoplasm. Albert's stain, a differential stain, stains the granules black, does not stain the bands, the stains the balance of the cell green. It is used to differentiate granulated *Corynebacterium* (*C. diphtheriae* and *C. xerosis*) from other corynebacteria. Further tests can then be used

to differentiate extremely virulent *C. diphtheriae* from the nonpathogen, *C. xerosis*.

(2) *Reagents:*

(a) Metachromatic Stain R-S (44).

(b) Mordant R-S (48).

(3) *Technique:*

(a) Prepare a thin-film smear, air-dry and heat-fix.

(b) Flood with staining solution for 3 to 5 minutes.

(c) Wash in water; drain and *blot dry*.

(d) Flood with iodine solution for 1 minute.

(e) Wash, drain, blot dry and examine.

(4) *Interpretation.* Metachromatic granules stain black, banded areas are blue to blue-green, and the balance of the bacterial cell stains green.

(5) *Discussion:*

(a) The iodine solution is the same as the Gram-iodine used in the Gram stain procedure.

(b) The stain procedure does not identify *C. diphtheriae*. It must be used as only one of several steps in the identification process.

(c) Several Gram-negative organisms have granules; therefore, the Gram stain must be done prior to the Albert stain to confirm that the organism is a diphtheroid.

(d) Smears should be made from slants of Loeffler's medium.

k. **Methylene Blue Stain:**

(1) *Principle.* The dye, methylene blue, possesses polychrome staining characteristics; components in the dye vary in color from purple to pink. When the stain is applied to a bacterium possessing dense granules within its cytoplasm, the granules stain darker than adjacent areas of the cell. The dense-staining areas are called metachromatic or volutin granules.

(2) *Reagent.* Methylene Blue R-S (45).

(3) *Technique:*

(a) Prepare a smear from a Loeffler's medium slant, air-dry, and heat-fix.

(b) Flood with stain for 1 minute.

(c) Wash well but gently with running tap water.

(d) Drain, blot dry and examine microscopically.

(4) *Interpretation.* Granules appear dark blue. The balance of the cell is lighter blue in color.

(5) *Discussion:*

(a) High quality dye should be used. Certified dyes with a color index (CI) number assigned by the US Commission of Biological Staining are recommended.

(b) Stain, prepared as above, is a satisfactory substitute for Loeffler's methylene blue stain.

(c) Methylene blue, as formerly prepared and used to stain granules, contained acid components that required neutralization with alkali. Presently certified methylene blue is much purer, and no alkali is required in making a satisfactory stain.

l. **Spore Stain (Wirtz-Conklin):**

(1) *Principle.* Bacterial spores are difficult to stain. In smears stained by ordinary methods, they appear as unstained gaps or holes within the cytoplasm of bacillary cells. Free spores may appear as tiny blue, brown, or red rings, for only the outer shell of the spore is faintly stained. Bacterial spores are more readily demonstrated by special staining methods which consist of steaming the dye-covered smear on a glass slide for a brief period. Staining is effected by lowering the surface tension between the spore coat and the dye solution. Once the spore is stained, the vegetative portion is counterstained with a dye of contrasting color.

(2) *Reagents:*

(a) Malachite green stain R-S (41).

(b) Safranin counterstain R-S (61).

(3) *Technique:*

(a) Flood the heat-fixed smear with malachite green and gently steam for 3 to 6 minutes by passing a flame under the flooded slide on a staining apparatus. Avoid boiling and heating the preparation to dryness.

(b) Wash the slide thoroughly with tap water.

(c) Counterstain with safranin for 30 to 60 seconds.

(d) Wash the smear again and blot dry.

(e) Examine the smear using the oil immersion objective.

(4) *Interpretation.* The bacterial spores stain green, while the vegetative bacillary cells stain pink to red.

m. **Stain of Blood Smears, Exudate, etc.** Smears of blood and exudates or tissue impressions are sometimes prepared and examined for *Borrelia recurrentis*, *Bacillus anthracis*, or other organisms, using Wright's or Giemsa's staining technique. The stains are available as stock items with directions for proper preparations included. (See AFM 160–51, *Clinical Laboratory Procedures—Hematology.*)

(1) *Giemsa Stain.* In staining a thin or thick smear, cover it with 15 drops of Giemsa

and let stand for 60 seconds; then add 30 drops (1.5 cm^3) of buffer solution, mix well with the dye solution and let mixture stand for 5 minutes. Wash the slide by flooding with tap water; allow to dry at room temperature. Examine it microscopically using the oil immersion objective.

(2) *Wright's Stain.* Wright's is primarily used in hematology but on occasion is useful in staining blood smears for bacterial detection. Flood the fixed smear with stain, add 2 to 3 drops of buffer, and allow the slide to stand for 3 to 5 minutes. Wash and allow to dry. Examine the slide for the presence of bacteria using the oil immersion objective.

3–8. Preparation of Wet Unstained Mounts for Microscopic Examination. Drops of fluid containing organisms are often examined advantageously as unstained preparations, referred to as wet mounts. This type of preparation is a fast, reliable method of determining selected morphological characteristics and motility.

a. **Detecting Morphological Characteristics.** These preparations are most often used to differentiate spiral bacteria from other forms. Syphilis, a venereal disease, can be presumptively diagnosed if spiral forms are found in characteristic sores called chancres, associated with the disease. Spiral bacteria cannot be stained with ordinary staining methods. (exceptions: *T. vincentii, B. recurrentis*). However, their presence can be detected easily by mixing a drop of saline and examining the preparation with a dark field microscope. Darkfield is used almost exclusively to investigate chancres for the presence of *Treponema pallidum* (causative agent in syphilis). Darkfield illumination is described in chapter 11.

b. **Detecting Motility.** The wet mount in the hands of an experienced worker is the fastest method of determining if organisms are motile or not. Motility is an important characteristic to determine, the bacteriologist can make decisions as to what tests, media, incubation temperatures, or other means must be used to determine motility. True motility must be differentiated from *Brownian movement.* When microorganisms or other similar-sized particles in colloidal suspension are looked at microscopically, slight apparent movement will always be seen. This type of movement, called brownian movement, is due to collision of particles with molecules of the surrounding medium. In Brownian movement, the particles tend to oscillate continuously but do not show directional movement. True motility results in directional movement, and a motile organism will frequently move from one side of the microscopic field of view to another. Although flagella that cause this movement will not usually be seen, locomotion will usually be obvious. The preparation can be examined with the darkfield or phase microscope if desired.

3–9. Coverslip Wet Mount. In the coverslip, wet-mount preparation, a drop of liquid specimen is sealed between a glass slide and coverslip. The preparation is prepared as follows:

a. Rim a clean, acid-washed coverslip with petrolatum. This can be best accomplished by smearing a small amount of the grease on the butt of the palm of the hand. The sealant is carefully scooped off the palm until an edge of grease has been built up around all four sides of the coverslip.

b. Place a loopful of specimen and a loopful of saline in the center of the coverslip and mix.

c. Cover the droplet with a clean, acid-washed slide, pressing the slide down gently to make a firm contact with the coverslip, forming a thin film sealed between the slide and coverslip.

d. Turn the right side up and examine microscopically under reduced light.

e. The coverslip should be completely sealed around its entire edge. This prevents evaporation of the fluid, contamination, etc.

3–10. Hanging-Drop Mount. In the hanging-drop preparation, a drop of liquid specimen on a coverslip is suspended over the concave well of a culture slide. The drop adheres to the coverslip but does not touch the culture slide. Prepare it as follows:

a. Ring a coverslip with petrolatum in the manner described in the preceding paragraph.

b. Place a loopful of specimen in the center of the coverslip. If necessary, a small amount of specimen can be mixed in a small drop of saline directly on the coverslip. Mixing must be carefully done to avoid spreading the droplet into a film.

c. Invert a culture slide, with concave well over the droplet of specimen, and press down firmly, sealing the coverslip to the slide.

d. Reinvert the slide and examine microscopically under reduced light.

e. The droplet of specimen should not touch the culture slide.

Chapter 4
BACTERIA CULTIVATION

4-1. Significance. Knowledge of cultivation methods is essential in bacteriology because it is basic to the identification of bacterial organisms. Samples of pathogens obtained in conjunction with the disease process are inoculated to various media and studies are performed on the growth obtained following media incubation. This necessitates an understanding of the growth requirements for the cultivation of bacteria.

4-2. Definition and Description. A culture medium is any compound or mixture which supports the growth and reproduction of bacteria. The medium provides an artificial environment for the laboratory study of bacteria. There are certain factors on which the development of the microorganisms depends: first, incubation of the organisms at the proper temperature throughout the growth period; second, proper gaseous atmosphere; third, the appropriate pH range of the medium; and lastly, the presence of suitable nutrients.

SECTION B—BACTERIAL GROWTH FACTORS

4-3. Temperature. Bacteria vary considerably with reference to temperature requirements. According to their growth at different temperature ranges, they can be separated into three groups: psychrophilic, mesophilic, and thermophilic.

a. **Psychrophilic.** Psychrophilic bacteria grow at relatively low temperatures (5°C to 30°C).

b. **Mesophilic.** Mesophilic bacteria grow best at a temperature close to that of the human body (30°C to 35°C). The majority of human pathogens are mesophilic, growing best at approximately 35°C. A constant temperature incubator, adjusted to 35°C and containing sufficient moisture, satisfies the temperature requirement for good growth of bacteria in the laboratory.

c. **Thermophilic.** Thermophilic bacteria grow best at a higher temperature range (50°C to 60°C).

4-4. Gaseous Environment. It is essential that the proper gaseous environment be furnished when attempting to cultivate bacteria. The specific needs apply to oxygen and carbon dioxide. Failure to isolate a pathogenic bacteria is often due to inadequate provisions for aeration.

a. **Oxygen Requirements.** Bacteria are divisible into four broad groups on the basis of their oxygen requirements:

(1) *Obligate Aerobes.* An obligate aerobe MUST have free atmospheric oxygen in order to grow. These forms grow best on agar media in a normal oxygen tension. They use atmospheric oxygen as a source of energy as well as that resulting from carbohydrate metabolism. Some members of the genus *Bacillus* are *obligate aerobes* which grow in the very top of tubed liquid media, where the oxygen content is highest.

(2) *Microaerophils.* Organisms in this category grow best at low oxygen tension, such as that supplied in the lower portion of a broth medium. The organisms also grow well on an agar plate, when incubated in an environment of increased carbon dioxide and low oxygen (candle jar). *Haemophilus* spp and many streptococci are microaerophilic organisms.

(3) *Facultative Anaerobes.* Organisms capable of growth in the presence or the absence of environmental oxygen are termed facultative.

(4) *Obligate Anaerobes.* Organisms which are unable to thrive in the presence of oxygen (free or atmospheric) are termed obligate anaerobes. If oxygen is used by these organisms, toxic byproducts are formed, which are lethal for the bacteria. The toxicity is largely attributed to oxidation of key enzymes vital to the existence of the organism. This prevents the completion of processes necessary to the survival of the organism. Therefore, special measures must be taken when culturing for anaerobic organisms to exclude or remove oxygen, thereby increasing the likelihood of recovery of the organism. Examples of obligate anaerobes are found in the species of the following genera: *Clostridium, Peptostreptococcus, Bacteroides,* and *Fusobacterium.*

b. **Anaerobic Methods.** There are many methods by which anaerobic conditions may be simulated for growth of bacteria which are unable to thrive in an environment containing either free oxygen or molecular oxygen. A method of providing anaerobic conditions for culture was introduced in 1915 by Laidlow. His principle on anaerobiosis led to the devel-

opment of many similar apparatuses in the years that followed. The principles basically involved the use of a tight lid container from which oxygen was removed by various means including the use of a catalyzed chemical. Modification of the process led eventually to the use of the cold catalyst (palladium-coated alumina pellets), which requires no heat to be activated. The Brewer modification of McIntosh and Fildes process used an electrical source to activate the catalyst (figure 4–1). A possibility of a hydrogen explosion always existed in this process if the system was in any way faulty. The coated alumina catalyst removed the need for using hazardous methods. Other ways to provide anaerobic conditions are (1) chemical absorption of oxygen (Buchner); (2) replacement of air by inert gas; (3) removal of oxygen by combustion (McIntosh and Fildes); and (4) thioglycollate medium.

(1) *Chemical Absorption of Oxygen.* Anaerobic conditions are produced by absorption of oxygen using pyrogallic acid and sodium hydroxide. Place powdered pyrogallic acid in a beaker in a dessicator jar and arrange the culture plates along side of the beaker (figure 4–2). This allows for free access to the beaker. Add sodium hydroxide (NaOH) solution (1 Normal) by means of a pipette to the pyrogallic acid and then immediately cover the dessicator with the lid. Stopcock grease should be used to insure a tight seal. The jar is then placed in the incubator. The amounts of pyrogallic acid and NaOH solution required will vary as the dessicator volume changes. The required amount of each ingredient is approximately 2 gm of pyrogallic acid and 10 ml NaOH for each 100 cm^3 of volume in the vessel.

(2) *Replacement of Air.* Replacement of air by inert gas is most effective using an anaerobic jar. Plate or tubed medium can be used with this jar. Two hose connections are provided: one inlet hose reaches the bottom of the jar; the other hose is the outlet. Air is exhausted from the jar by means of a suction pump attached to the outlet hose. The outlet is provided with a two-way stopcock, which is also connected to a gas mixture, as shown in figure 4–3 (80 percent N_2, 10 percent CO_2, 10 percent H_2, which is available commercially). After all air has been removed, the gas mixture is allowed to flow into the bottom of the jar until the air has been replaced. A gauge is attached to the system so that the vacuum can be measured; when the replacement of gases occurs, the indicator needle will move back to zero. A mercury manometer can be used instead to monitor the gas exchange process.

(3) *Removal of Air by Chemical Displacement.* The Gas Pak is a modification of methods devised by McIntosh and Fildes, Brewer, and others. Cultures are sealed in a jar along with a hydrogen generator and an indicator strip. The hydrogen generator envelope must be charged with 10 ml of distilled water prior to sealing the jar (figure 4–4). The catalyst in the reaction is palladium-coated alumina pellets. The pellets should be changed weekly in

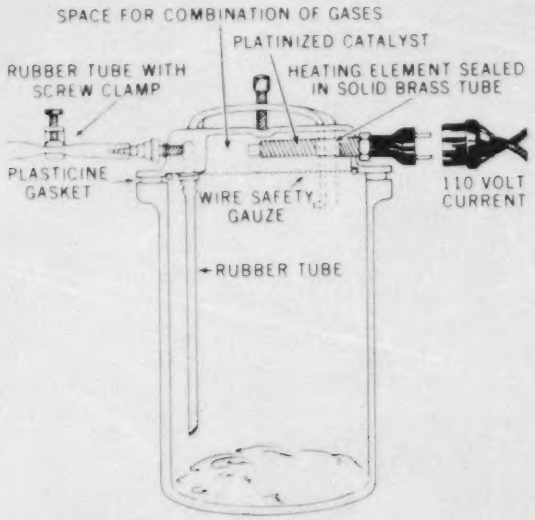

Figure 4–1. Brewer's Jar for Anaerobiosis.

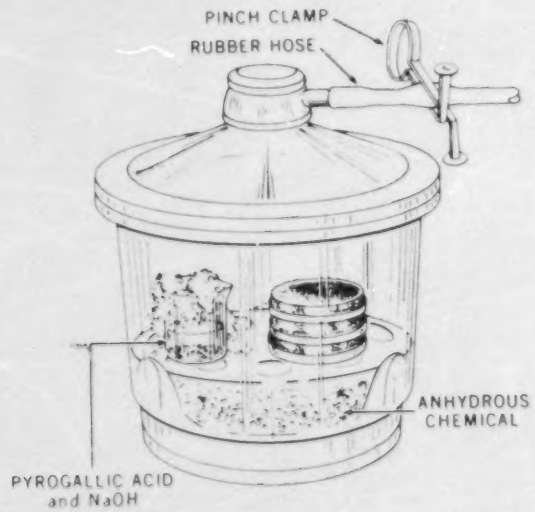

Figure 4–2. Dessicator Jar/Pyrogallic Acid.

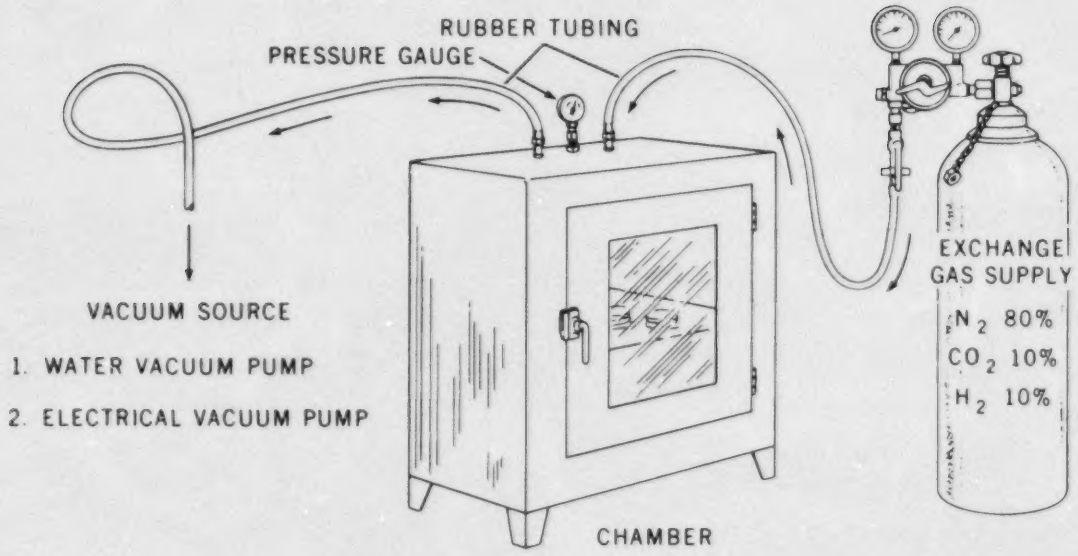

Figure 4–3. Anaerobic Incubator Using Bottle Gas.

that they are inactivated by dampness. However, by heating the used pellets at 160°C for 2 hours in a hot-air oven, they can be reactivated. The steps of the Gas Pak technique are as follows:

(a) Place catalytic pellets in the lid of the jar.

(b) Place the material to be incubated and the indicator strip (exposed) in the jar.

(c) Cut the corner off a Gas Pak hydrogen and carbon dioxide generator envelope, spread the foil open, and place it in the jar in an upright position.

(d) Pipette 10 ml of water through the opening into the envelope, taking care not to insert the pipette.

(e) Quickly place the Gas Pak lid on the jar. Place the clamp on the jar and secure it until tight.

(f) Place the jar in the incubator.

(4) *Thioglycollate Medium Method.* The use of fluid thioglycollate is the simplest of the methods mentioned above for providing anaerobic conditions (Brewer, 1940). This medium will support growth varying from aerobic to strict anaerobic conditions. For the cultivation of anaerobes, the medium should be heated in a boiling water or steam bath to expel the absorbed oxygen, then cooled prior to inoculation. The medium should not be reheated more than once. Routine use of thioglycollate with all specimens obtained from wounds is highly recommended.

c. **Carbon Dioxide Requirements.** Many microorganisms require a carbon dioxide concentration above that of the normal environmental level. Three ways of providing an increased carbon dioxide enviornment are as follows:

(1) *Candle Jar Method.* An environment containing 2 to 3 percent carbon dioxide is adequately supplied by incubating cultures in a candle jar (see figure 4–5). Any wide-mouth vessel with an airtight lid will suffice, providing it is over 4 inches in diameter across the opening.

(a) Place inoculated tubes or plates of media in the vessel.

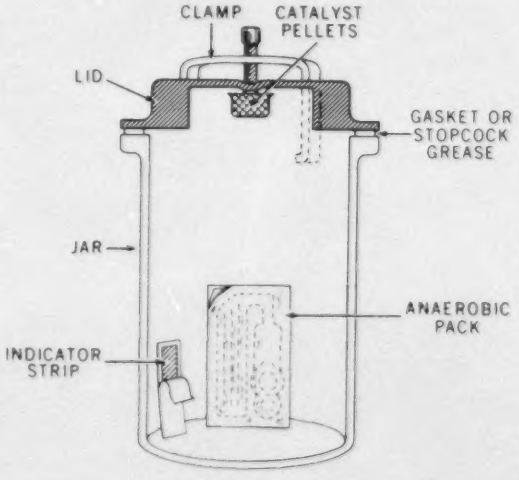

Figure 4–4. Gas Pak Anaerobiosis.

(b) Place a lighted candle in the container with the cultures.

(c) Tighten the lid securely and place the jar in the incubator.

(d) Place the candle as high as possible in the jar to permit accumulation of carbon dioxide, which extinguishes the flame. The candle flame will be extinguished when about 2 to 3 percent carbon dioxide has accumulated.

(2) *Sodium Carbonate Method.* When 10 percent carbon dioxide is required, the following method may be employed:

(a) To a small container in the jar, add 1 gram of sodium carbonate for each 2.5 liters of vessel capacity.

(b) Cover the sodium carbonate with cotton.

(c) To this, add 100 milliliters of 3 percent sulfuric acid for each gram of carbonate, and quickly secure the lid tightly on the jar.

(d) Place the container in the incubator for the incubation period.

(3) *"Ammo-Box" Method.* An alternate method of providing carbon dioxide for growth has been suggested, utilizing a modified 30-caliber ammunition box. The technique using the "ammo-box" is very simple. Note the gauge on top of the box, as shown in figure 4–6. This gauge adds 5 pounds of CO_2 (carbon dioxide) to the cultures using a regular CO_2 cylinder, as illustrated in figure 4–6.

(a) Place the cultures in an incubator overnight for growth.

(b) Prior to removing the plates from the box, check to see whether the system is still under pressure.

(c) If so, simply release the pressure by pressing the valve needle of the inlet.

(d) Figure 4–6 illustrates the use and technique of this method. The apparatus is proposed as an easy means of preparing an inexpensive CO_2 chamber. Any other setup using this basic concept (increased CO_2 tension to the plates) will suffice.

4–5. pH (Hydrogen-Ion Concentration). The growth of microorganisms is markedly affected by the pH of the medium. Most bacteria, especially pathogenic forms, grow best near the neutral point (pH 7). The pH must be adjusted within proper range prior to sterilization.

4–6. Nutrients:

a. **Basic Requirements.** There is no single medium satisfactory for cultivating all bacteria. However, there are basic requirements which are fundamental to most media. A basic

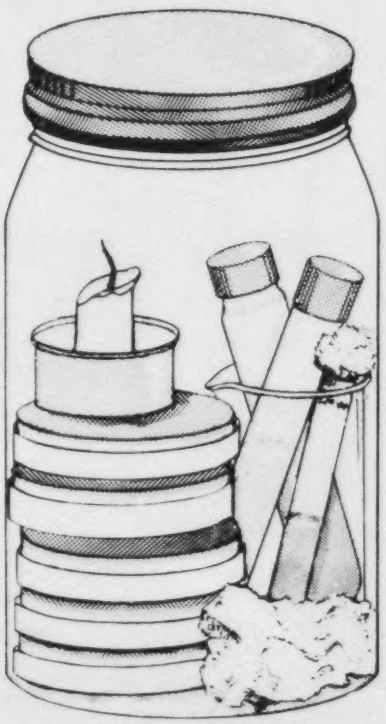

Figure 4–5. Candle Jar/Increased Carbon Dioxide Tension.

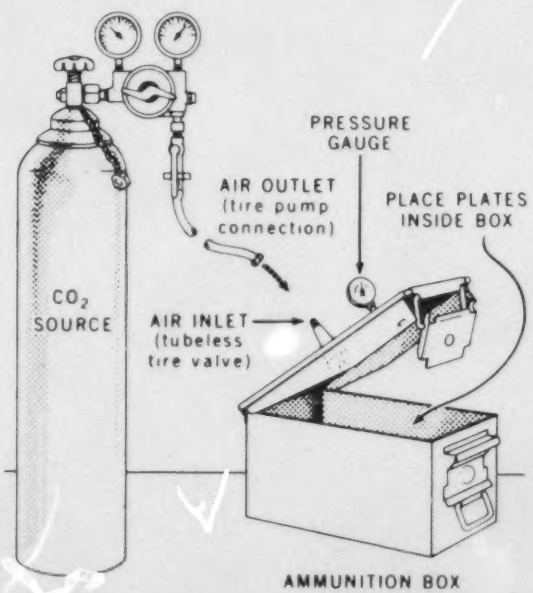

Figure 4–6. Ammunition Box/Modified CO_2 Chamber.

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culture medium requires carbon sources, nitrogen, and inorganic salts. Other substances, such as blood, serum, amino acids, or vitamins, may be required. Other complex energy sources may include extracts or infusions of meat, vegetables, or yeast, hydrolysates, or protein digests, which yield amides, amino acids, peptides, peptones, and proteoses. Consult attachment 2 of this manual for media preparations.

b. **Carbohydrates.** Carbohydrates, alcohols, organic acids, glucosides, and other organic acids, glucosides, and other organic compounds provide additional sources of energy for cultivation of bacteria. Carbohydrates, especially glucose, are sometimes incorporated in media used for the primary isolation of pathogens.

SECTION C—MEDIA

4–7. Culture Media:

a. **Liquid Media.** Media in liquid form are known as broths. Bacterial cells are able to multiply and move with intermixing in a liquid medium.

b. **Solid or Gelatinous Media.** Bacteria often occur in specimens in association with other bacteria. In order to identify any one bacterium accurately, it is necessary to isolate it in a pure state. Pure cultures are best obtained by using solid medium. If a small number of individual cells are deposited on a solid medium, each cell will be immobilized and will multiply to form a pure, visible colony. Liquid media or broths may be gelled by the addition of agar, a purified extract of Gelidium or seaweed (1.5 percent w/v). The procedure for adding agar to a liquid medium follows (preparation of media is discussed in more detail in paragraph 4–8 and in attachment 2 of this manual).

(1) Add 1.5 percent w/v agar to a liquid nutrient medium.

(2) Melt the agar at a temperature range of 96°C to 98°C to bring it into solution.

(3) Cool the solution to 40°C) it will then solidify, and the medium is ready for use.

(4) Sterile conditions should be maintained during this procedure.

(5) When media are not to be poured immediately, they may be placed in a 56°C waterbath for a few hours until such time as they are poured.

c. **Semisolid Media.** Semisolid media using less than 1.5 percent agar are ideal for performing motility checks of pure cultures.

4–8. Preparation of Media. One important prerequisite in medium preparation is to read and follow the manufacturer's directions. This practice can save countless hours should anything go wrong and the medium not work properly. It will be easier to retrace steps in arriving at a solution to the problem. The preparation of medium invoives several steps. If the medium is in bulk powder, it has to be measured. It can be purchased in premeasured packets, but the bulk form is less expensive and requires only a short time to measure. The next steps in preparation of medium are rehydration, sterilization, and dispensing of the finished product.

4–9. Rehydration of Medium. Medium powder which is to be rehydrated must be carefully weighed out in the amount specified on the label or in proportional amounts when smaller portions are required. Paper cups are excellent for weighing the powder, and can be discarded after use. Rehydration requires the freshest water available for the best results. Contamination is no problem because the medium is generally sterilized prior to use. Fresh, warm, distilled water is a good choice because the powder goes into solution without additional heating and mixing. The steps in rehydration are as follows:

a. Measure the water and pour it into a flask twice the volume of the medium to be prepared.

b. Swirl the flask containing water while adding the powder so that the powder will go into solution. If a magnetic stirrer is available, it may be used to mix the solution.

c. Some magnetic stirrers are equipped with a heating element designed for use in medium preparation. If the solution is to be heated, care should be taken not to scorch the medium. Liquid media which do not contain agar are easier to prepare because they dissolve easily.

4–10. Sterilization Methods. Bacteriological identification requires the study of microorganisms in pure culture. Since microorganisms are found in practically all places, materials used in their cultivation must be sterilized prior to use. The fundamental purpose of sterilization is to remove or destroy all living organisms on or within a medium without rendering it ineffective for cultivating the desired microorganisms. Sterilization of materials can be accomplished in one of several ways, depending upon the need. Various ways of sterilization are (1) steam under pressure) (2) flowing steam (tyndallization); (3) inspis-

sation; (4) filtration; (5) chemical; and (6) dry heat. Steam under pressure is perhaps the most commonly used of the above-mentioned methods.

4–11. Steam Under Pressure. Pressurized steam is achieved through use of an autoclave. Since the steam is applied under pressure, it is very effective becausetemperatures are higher and penetration more through (see Table 4–1). Microbes are destroyed through denaturation or protoplasmic coagulation. It is the temperature reached with the aid of pressure which is the important factor in this process. All air must be replaced by steam in the sterilizing chamber of the autoclave before effective temperatures can be reached. Two factors should always be kept in mind when using the autoclave: the size of the load to be placed in the chamber and the exposure time. Autoclaves exist in many and varied types; therefore, a generalized approach to operation will be presented, applicable to conventional types of laboratory autoclaves.

a. **Operation of Autoclave:**

(1) Inspect the autoclave's interior for general cleanliness.

(2) Open the steam supply, and allow the jacket pressure to reach 15 to 17 psi.

(3) Place the materials to be autoclaved in the chamber and secure the door (new doors contain pressure locks).

(4) Turn the operating valve to the STERILIZE position, which allows steam to enter the chamber.

(5) When the discharge thermometer registers 121°C (250°F), the sterilizing process has started.

(6) Set a timer to indicate the end of the sterilization cycle.

b. **Removal of Materials From Chamber:**

Table 4–1. Pressure Temperature Relationships in the Autoclave.

Temperature in degrees Centigrade	Steam Pressure in Pounds
108	5
116	10
121	15
127	20
134	25
141	30

(1) *Nonliquid Materials:*

(a) Turn the operating valve to the EXHAUST position and then open it slowly until the chamber gauge registers zero.

(b) Turn the operating valve to VACUUM for exactly 3 minutes and then turn it to OFF.

(c) When the chamber gauge registers zero, unlock the door and loosen it just enough to allow vapor to escape (do not open it completely). Leave it in this position for 5 to 10 minutes, depending upon the size of the load.

(2) *Liquid Materials:*

(a) Leave the operating valve at the STERILE position; close the steam supply valve.

(b) When the chamber gauge registers zero, open the door and remove the load.

c. **Sterilization Check.** A steriliation check should be performed periodically. Commercial test kits are available in the form of spore strips. These strips are impregnated with *Bacillus stearothermophilus* and sealed separately in glassine envelopes (Kilit spore strips or ampules, BBL). The steps in the sterilization check are as follows:

(1) Place two test strips in appropriate sections of the chamber when the autoclave is loaded.

(2) After the sterilization cycle is completed, place the test strips along with a control strip aseptically into separate tubes of thioglycollate broth.

(3) Allow these strips to incubate for 7 days at 56°C.

(4) Normal growth of *B. stearothermophilus* developing from the control strip, together with absence of growth from either of the test strips, indicates that the sterilization procedure was efficient.

d. **Points to Remember.** The autoclave is a potentially dangerous apparatus; check it periodically to make sure that it is in proper operating condition. Use the following pointers:

(1) Never overload the autoclave.

(2) Allow the autoclave to preheat before using.

(3) Never overautoclave or reautoclave media.

(4) Never fill the media flask to more than two-thirds of its capacity.

(5) Remove all media from the autoclave as soon as possible. If media cannot be poured immediately, place it in a 56°C waterbath for holding.

(6) At 121°C, 15 minutes should be suffi-

cient time to routinely sterilize most items used in the clinical laboratory.

(7) Don't fast-exhaust liquids.

(8) Periodically clean the plug drain and flush the drain line with hot trisodium phosphate solution.

(9) Periodically wash the loading car (or inside rack)und inner chamber with a sanitizing solution.

(10) Maintain quality control (see c above, Sterilization Check).

4–12. Tyndallization (Flowing Steam). This method of sterilization was named after John Tyndall, who is credited with its introduction. To sterilize by this method, subject the materials to 100°C temperatures on 2 or 3 successive days. The first exposure kills the bacterial forms and vegetative forms, leaving the spore forms. On the next heating, those spore forms will have germinated and will be killed. The process can be continued into the third day for better results. This method is of value when an autoclave is not immediately accessible.

4–13. Inspissation. This method sterilizes through an evaporative thickening of proteins in materials that cannot be subjected to heat, such as serum in Loeffler's medium or egg in Lowenstein-Jensen medium. An inspissator or sterilizer may be used in this procedure. Materials to be sterilized are placed in tubes in a slanted position and subjected to 60°C to 80°C for 30 to 60 minutes on 4 to 7 consecutive days. Although this procedure is useful with substances which may be otherwise destroyed by heat, it is not a reliable method of sterilization.

4–14. Filtration. Filtration is useful as a method of sterilization for materials which would be altered at high temperatures. Materials often used in media, such as urea, serum, plasma, or certain carbohydrates, are among those which should be filtered rather than heat-sterilized. Filters for sterilizing are usually constructed of unglazed porcelain, compressed asbestos, or membranes capable of filtering bacterial substances:

a. Place the filter material in a sterile holder and place it on a flask so constructed that a vacuum source may be applied.

b. Fill the side vent with cotton and cover the flask with an appropriate material prior to sterilizing the apparatus.

c. After sterilization and cooling, connect a suction or vacuum pump (negative pressure) to the sidearm.

d. Place the material to be sterilized in the holder and apply suction.

e. Check sterility of materials by overnight incubation, and inspect for growth the following day.

4–15. Chemical Sterilization. In strict application of the term "sterilization," chemical sterilization is not possible. However, if the term "chemical disinfection" is used, it can be stated that by the use of chemicals we "sterilize" (disinfect) by using chemicals which are lethal to microorganisms. Two very familiar disinfectants used in bacteriology are thymol and phenol. Phenol can be used in strengths of 2 to 3 percent for general control of surface bacteria in working situations. Thymol crystals can be used to "sterilize" urea that is 20 times its normal concentration, or a 20 percent carbohydrate solution. This would require 1 gm of thymol crystals per 100 ml of solution in each case. The materials are then allowed to stand 24 hours at room temperature. After 24 hours, the solutions may be diluted for use; dilution serves to cancel further effects of the thymol.

4–16. Dry Air Sterilization. Dry air sterilization is accomplished in the absence of steam heat. Dry air ovens used to dry laboratory glassware are excellent for this type of sterilization. Materials not heat destructible can be sterilized by exposure to 160°C for 2 hours. The mode of action in this process is oxidation of intracellular constituents by heat. Sterilization can be accomplished by the following regimens:

172°C (342°F) for 1 hour.
160°C (320°F) for 2 hours.
121°C (250°F) for 6 hours or longer.

It is important not to overload the oven because overloading requires increased exposure time. Rubber goods and some plastics should not be sterilized by this method. The dry air sterilizing method is often used for cotton-plugged tubes, pipettes, and other nonliquid materials.

4–17. Dispensing Media. Media for general use in bacteriological identification are prepared and dispensed in whole plates, triplates, biplates, and test tubes. Dispensing a uniform amount of medium in each instance is desirable. With uniform dispensing, the number of plates or tubes can be accurately determined without appreciable waste of media. This is helpful also when you have a limited number of

vessels to fill. Four methods of dispensing media will be presented: the continuous media dispenser, the inverted flask, manual pouring, and the automatic syringe.

a. **Continuous Media Dispenser.** Continuous dispensers are usually electronic and can be adjusted to deliver the desired amounts of media routinely required in the laboratory. This method is excellent for preparing bi- or triplates when two or three different media are required. Another feature of the continuous dispenser is that only a flexible tubing or detachable part requires sterilization. Sterilization of these parts may be accomplished in advance.

b. **Aspirator Bottle Method.** The aspirator bottle is an excellent method for dispensing media. The technique requires an aspirator bottle and the dispensing tube to be attached and sterilized, as shown in figure 4–7. Be sure that all airways are covered prior to autoclaving. The setup for dispensing the media is shown in figure 4–8.

(1) Attach a 1-foot length of surgical tubing to the spout of a 4-liter aspirator bottle.

(2) Attach a 1½-inch piece of fine polished glass tubing (a section of 5-ml serological pipette may be used instead) to the free end of the tubing.

(3) Slide a small pinch clamp over the tubing.

(4) Wind some wire around the top of the bottle to serve as a hook to hold the spout while autoclaving.

(5) Wrap the top of the aspirator bottle and the spout tip in aluminum foil while autoclaving.

(6) The sterile media is poured into the sterilized aspirator bottle and dispensed by regulating the flow with the pinch clamp.

c. **Manual Pour.** Pouring media by hand is practical when small quantities of special media are prepared to perform tests at infrequent intervals. When pouring by hand it must be remembered that contamination is possible and flaming the flask neck is important.

d. **Automatic Syringe.** The automatic syringe is best suited for dispensing liquids for slant cultures and other biochemicals. Plates may be prepared with the syringe, but sterility must be kept in mind. In the preparation of tubed media, the medium is sterilized after it has been dispensed, so sterilization of the syringe is not a necessity (see figure 4–8).

4–18. Classification of Media. In order to select and prepare culture media for various bacteriological analyses, it is necessary to become

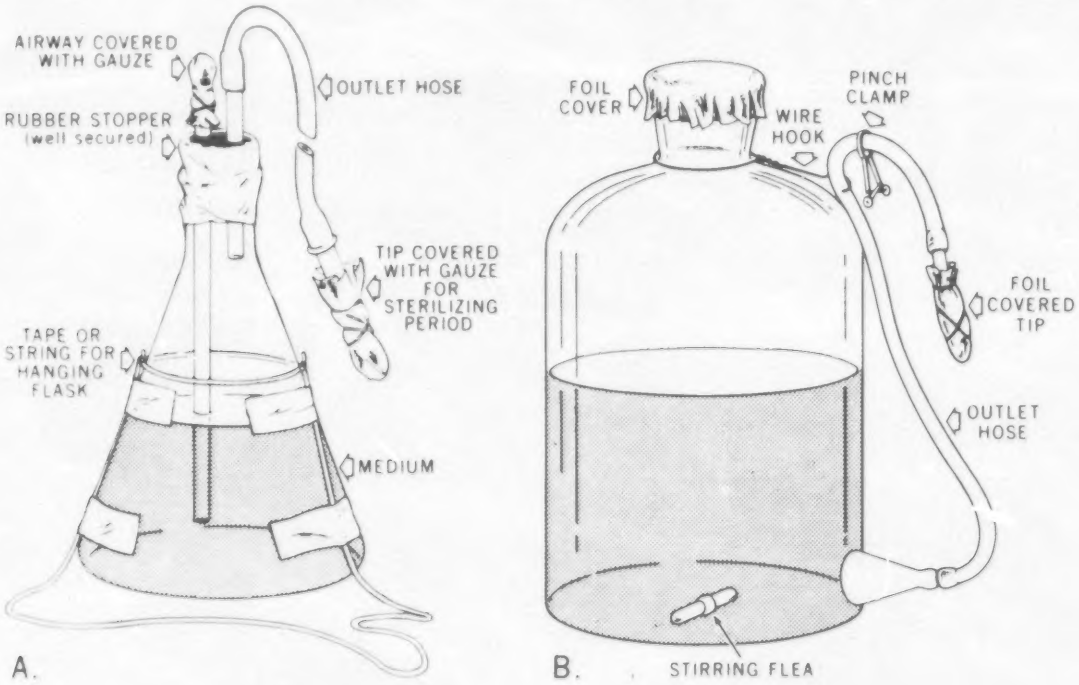

Figure 4–7. Aspirator Bottle/Media Dispenser.

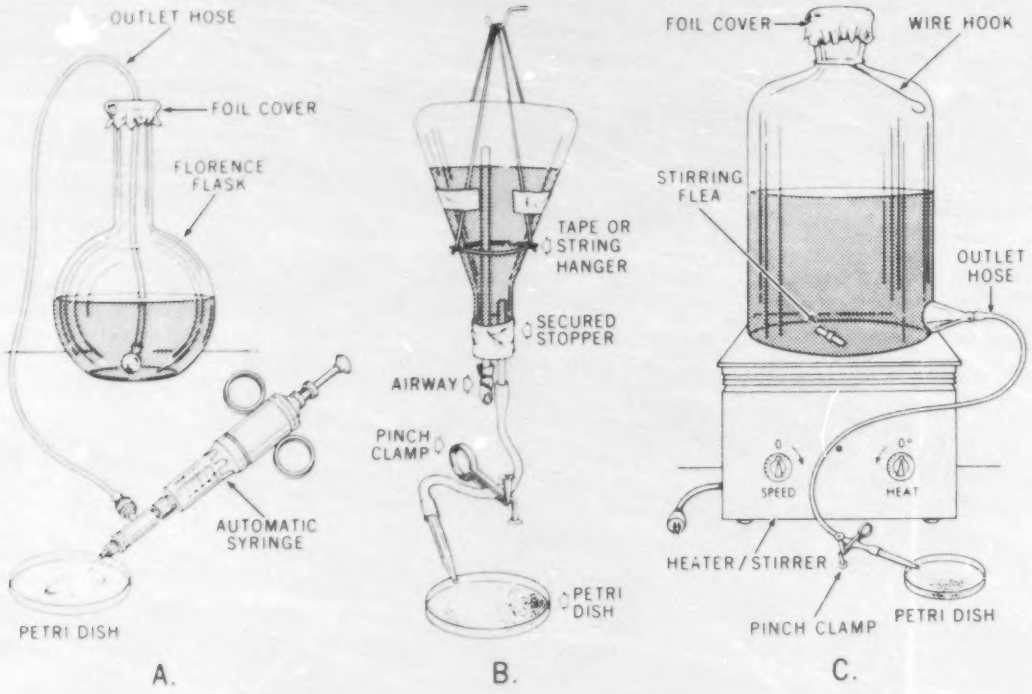

Figure 4–8. Media Dispensing Apparatus.

familiar with the many and varied types of media used. For the sake of convenience, media are classified according to the purposes for which they are generally used. The preparation and use of various kinds of media are described below.

a. **Basal Media.** Basal media contain ordinary peptone, peptone-meat extract, or peptone-meat infusion with 0.5 percent sodium chloride. Agar is added (1.5 to 2 percent) when solid media are required.

b. **Enriched Media.** These media are usually prepared by adding blood, serum, ascitic fluid, amino acids, or other complex nutrients to a basal medium for primary isolation and subculture of fastidious organisms. Since the additives are usually thermolabile or destroyed by heat, sterile enrichment substances must be added after sterilization and cooling (50°C) of the basal medium.

c. **Isolation Media.** These media are used to obtain pure growths of pathogenic microorganisms from a heavily contaminated source, such as fecal specimens. To accomplish this, specific agents are added to the media to retard or prevent growth of miscellaneous contaminants present in the specimen without appreciably affecting the growth of any pathogens present. When such media are employed in the solid state, specific carbohydrates and acid-base indicators may be added to enable gross differentiation between organisms on the basis of fermentation reactions. Isolation media are subdivided into differential, selective, and inhibitory media (described in chapter 8).

d. **Biochemical Media.** Biochemical media are employed to detect enzyme activity of bacteria in pure culture. The ability of various organisms to hydrolyze urea to ammonia and carbon dioxide, ferment various carbohydrates to acid and gas, reduce nitrates to nitrites, or produce indole from tryptophan are all examples of enzyme activities which are useful in differentiating bacteria. Any medium which will enable the detection of these reactions or comparable reactions may be termed a biochemical medium.

e. **Media Containing Antibiotics.** Since various antibiotics in appropriate concentrations are inhibitory to some organisms and noninhibitory to others, they may be used in the selective isolation of certain pathogens from specimens containing contaminating microorganisms. For example, penicillin may be incorporated in Bordet-Gengou agar in a concentration of 1 unit per milliliter of medium. The antibiotic will restrict growth of Gram-positive organisms of the throat and thus fa-

cilitate isolation of *Bordetella pertussis*. Penicillin and streptomycin in combination or chloramphenicol alone may be an additive to the medium for isolation of certain fungi from contaminated specimens.

4–19. Media Additives. There are many differ- reagents which can be added to media for special purposes. Some reagents neutralize antibiotics which might be present in the specimen submitted from an infection. Others promote growth of specific organisms having unusual nutritional needs and differential additives.

a. **Para-aminobenzoic Acid.** This substance neutralizes the bacteriostatic effect of sulfonamides in culture media at a concentration of 0.002 percent. This requirement pertains particularly to urine, blood, and fecal cultures from patients on sulfonamide therapy.

b. **Sodium Thioglycollate.** This compound is added, because of its reducing action, to permit anaerobic growth of bacteria but at the same time to neutralize the bacteriostatic effects of mercury and silver disinfectants. Mercury compounds are often used as preservatives in plasma, serum, biologicals, and pharmaceuticals intended for parenteral injection. Thioglycollate liquid medium is routinely used in most laboratories.

c. **Antibiotic Inhibitors.** These substances are added to culture media to neutralize the activity of antibiotics which may be present in specimens from patients under therapy; for example, the enzyme penicillinase to inactivate penicillin and also streptomycin to some degree. In culturing blood or other body fluids containing penicillin, add 1 ml of penicillinase to each 100 ml of sterile media. This amount of enzyme inactivates 50,000 units of penicillin, which is the highest level that could be expected in 10 ml of blood or other body fluids. One ml of penicillinase also inactivates up to 1,000 units of streptomycin. Penicillinase does not interfere with the sulfa-inactivating action of para-aminobenzoic acid, which also may be added to the medium. Penicillinase should be refrigerated when not in use, because enzyme activity is lost at room temperature.

d. **Dyes.** Dyes are incorporated in media to give them selectivity through bacteriostatic effects and are generally most active against gram-positive organisms. Crystal violet, brilliant green, and basic fuchsin are the most common selective dye media.

e. **Other Additives.** Other substances added to media are sodium desoxycholate, bile salts, potassium tellurite, and sodium azide. Sodium azide inhibits the growth of gram-negative organisms and can therefore be used to isolate gram-positive organisms.

SECTION D—ISOLATION OF BACTERIA

4–20. Overview. Bacteria usually occur as a mixed population, that is, not in a pure culture. Therefore techniques are necessary to isolate a specific bacterium for study. To secure a pure culture of a given organism from a specimen or sample containing mixed flora, it is necessary to isolate a single bacterium from all other bacteria present. The bacterium is cultivated in such a manner that its colonial progeny remain isolated. One of the following methods may be used to inoculate specimens or broth cultures to agar media to obtain isolated colonies.

4–21. Streak Plates. Streak plates are prepared on the routine solid culture media found in most laboratories. To obtain well isolated colonies from specimens, streak plates as shown in figure 4–9. Streak sterile loop containing inoculum over the plates, diluting the bacteria with each application. As the streaking continues, fewer and fewer bacteria remain on the loop until finally a single bacterium is deposited on the agar. Each isolated bacterium will give rise to a visible colony under suitable environmental conditions. The streak plate method is the most common means of securing isolated colonies and when done properly is as reliable as and requires less time than the pour-plate. methods.

4–22. Pour Plates. The pour-plate technique for securing isolated colonies is generally employed using blood agar.

a. Place culture tubes containing 12 ml of sterile infusion agar or other suitable media in boiling water to melt the agar.

b. After cooling the medium to 48°C in a waterbath, add approximately 0.7 ml to 1.0 ml of sterile, defibrinated blood aseptically to each tube of melted agar.

c. Inoculate each mixture with a sample of the appropriately diluted specimen or broth culture, mix well by twirling the tube, and dispense to sterile plastic culture plates. It is important that the inoculum be diluted in accordance with the concentration of the organisms observed in the stained specimen.

d. Upon solidification of the medium, individual bacteria in the inoculum are immobilized in various areas of the agar. Single visible colonies will form within the medium.

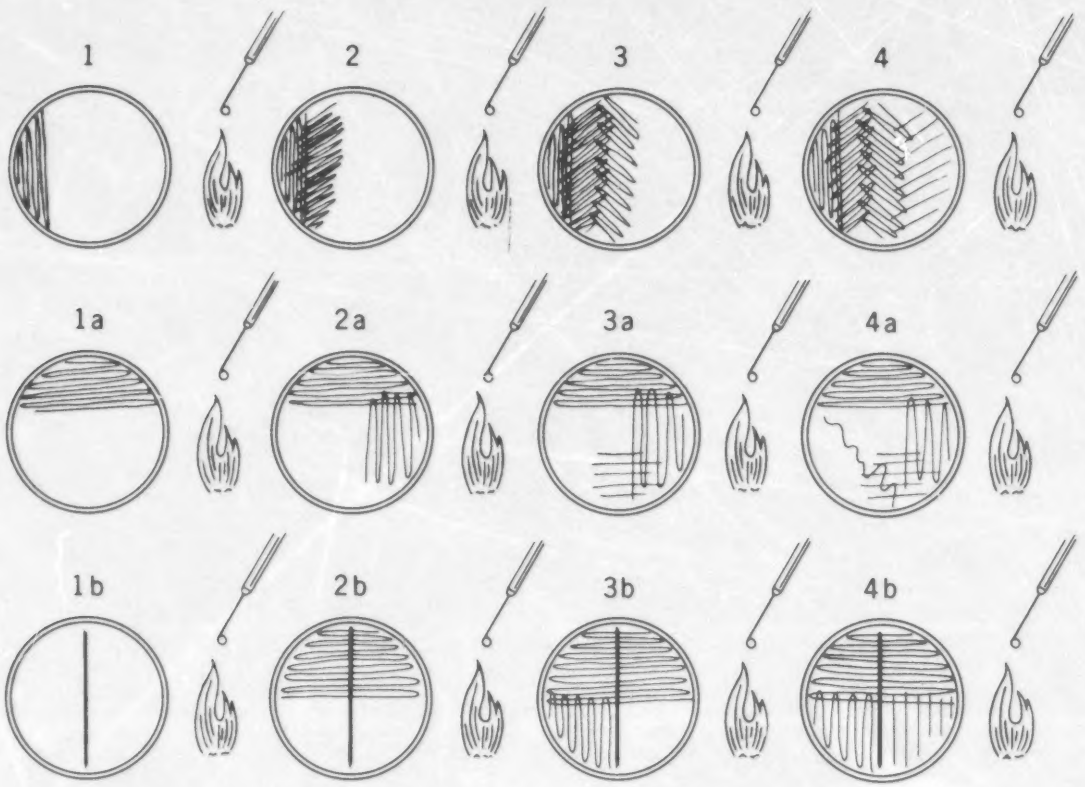

Figure 4–9. Three Examples of Isolation Streaks.

e. When subcultures or strains are to be prepared from plate cultures, it is necessary to secure growth from individual colonies within the agar using a sterile needle.

4–23. Test Tube Culture. After isolated colonies are obtained by the streak plate or pour plate technique, it is often necessary to subculture growth to tubed media by dispensing broth or agar media into appropriate test tubes. Plug the tubes with cotton or plastic sponges; screw-capped tubes also may be used. half placed in the tube and half extending out of the top. The plug should fit snugly, but not so tightly that difficulty will be experienced in removing and replacing the plug during routine manipulations. After sterilization, allow broth and agar stab tubes to cool in the upright position. Prepare agar slants by inclining the tubes of melted agar medium on a tabletop until solidification occurs. The three common tube media are inoculated by the methods described here.

a. **Liquid Cultures (Broth).** Using a sterile loop, emulsify a small amount of growth on the moist wall of the tube just above the liquid and wash down by tilting the tube. If the inoculum is liquid, simply place a loopful in the broth and disperse it by gentle agitation.

b. **Slant Cultures.** Inoculate slant cultures by streaking the inoculum over the slant containing water of condensation at its base. Do not spread the water over the surface of the slant since the resulting growth will not yield characteristic colony appearance.

c. **Stab Cultures.** Stab cultures are made with a straight needle into tubes of unslanted solid or semisolid medium. The stab line centered without lateral movement, should extend approximately one-half to two-thirds the depth of the medium.

4–24. Summary of Bacteria Encountered. Prior to proceeding to the chapters dealing with the isolation and identification of organisms, the following summary of various bacteria encountered is provided in figure 4–10.

ORGANISMS ENCOUNTERED IN THE BLOOD

Staphylococcus (coagulase negative and positive)
Coliform bacilli and related organisms
Alpha and beta streptococci
Pneumococci
Enterococci
Haemophilus influenzae
Clostridium perfringens
Pseudomonas species
Proteus species
Bacteroides species and related anaerobes
Neisseria meningitidis
Salmonella vaginicola
Brucella species
Pasteurella tularensis
Listeria monocytogenes
Herellea species
Streptobacillus moniliformis
Leptospira species
Vibrio fetus

ORGANISMS TO LOOK FOR IN THE THROAT

Beta hemolytic streptococci Group A
Corynebacterium diphtheriae
Meningococcus
Bordetella pertussis
PREDOMINANCE OF:
Staphylococcus aureus
Coliform bacilli
Pneumococci
Haemophilus influenzae
Candida albicans
Any other organisms predominating the flora

SPUTUM

Pneumococcus
Klebsiella pneumoniae
Coliform bacilli
Pseudomonas
Proteus species
Staphylococcus aureus
MYCOPLASMA PNEUMONIAE

RESPIRATORY TRACT INFECTIONS

Haemophilus haemolyticus
Haemophilus influenzae
Pneumococci
Nonhemolytic (gamma) streptococci
Diphtheroid bacilli
Coliform bacilli (particularly after penicillin therapy)

STOOLS

Salmonella spp.
Shigella spp.
Staphylococcus aureus
Mycobacterium tuberculosis (not routine)

Figure 4–10. Bacteria Routinely Encountered in Various Specimens.

URINARY TRACT

Escherichia coli
Klebsiella-Enterobacter-Serratia division
Proteus species and the Providencia species
Pseudomonas aeruginosa and other species of Pseudomonas
Enterococci (*Streptococci faecalis*)
Staphylococci coagulase positive and negative
Alcaligenes species
Haemophilus species (probably *H. vaginalis*)

GENITAL TRACT

Neisseria gonorrhoeae
Enterococci
Coliform bacilli
Bacteroides species (anaerobic)
Lactobacilli
Haemophilus vaginalis
Beta Streptococci Groups B and D
Haemophilus ducreyi

SPINAL FLUID

Haemophilus influenzae, type B (infants and children)
Neisseria meningitidis
Diplococcus pneumoniae
Mycobacterium tuberculosis
Staphylococci
Streptococci
Enterococci
Cryptococcus neoformans (fungi)

WOUNDS

Staphylococcus aureus
Streptococcus pyogenes
Coliform bacilli
Proteus species
Pseudomonas species
Clostridium species
Enterococci
Anaerobic cocci (Peptococcus Peptostreptococcus-Enterococcus)

EYES, EARS, MASTOID REGION & SINUSES

Staphylococcus aureus
Haemophilus species
Diplococcus pneumoniae
Neisseria gonorrhoeae
Corynebacterium diphtheriae
Pseudomonas aeruginosa
Coliform bacilli
Herellea species
Alpha and beta hemolytic streptococci
Moraxella lacunata
Aspergillus fumigatus

Figure 4-10. Continued.

Chapter 5
GRAM-POSITIVE COCCI

SECTION A—STAPHYLOCOCCI

5–1. Introduction. Organisms comprising the gram-positive cocci are the staphylococci, streptococci, and pneumococci. These bacteria are responsible for a variety of human infections. The infections range from relatively simple involvements of the skin and mucous membranes to more serious diseases, which may be manifested in pneumonia, septicemia, rheumatic fever, acute glomerulonephritis, or deep tissue abscesses. This chapter discusses some diseases caused by these bacteria along with some techniques involved in identification by laboratory methods.

5–2. Classification and Morphology:

a. **Taxonomy:**

Part 14.

Family I—Micrococcaceae
- Genus I—*Micrococcus*
- Genus II—*Staphylococcus*
- Genus III—*Planococcus* (*Gaffkya*)

Family III—Peptococcaceae
- Genus IV—*Sarcina*
- Genus I—*Peptococcus*
- Genus II—*Peptostreptococcus*

b. **Morphology:**

(1) All members of the family are gram-positive and catalase-positive. Cells are nonmotile, nonsporulating spheres, 0.5 to 1.5 μm in diameter.

(2) Staphylococci, micrococci, and peptococci are typically found arranged in "grape-like" clusters. This feature is useful in differentiating these organisms from members of the family *Streptococceae* which are also gram-positive spheres but are typically found arranged in moderate to long chains, with the exception of *Streptococcus faecalis*, which also exhibit clusters.

(3) Under certain conditions, staphylococci, micrococci, and peptococci will be found arranged in pairs and short chains.

(4) *Micrococci* typically are found arranged in tetrads and packets of eight cells. Colonies of growth resemble those of the staphylococci; some species are chromogenic, producing coagulase, and are nontoxigenic; for example, *Micrococcus lutea*.

5–3. Pathogenicity:

a. **Staphylococcus aureus** is generally considered the most common pathogen in the family. However, several other members, including *Staphylococcus epidermidis* and certain peptococci have been isolated from disease processes. The true role of such isolates is currently controversial.

b. **S. aureus** is isolated most often from diseased skin and mucous membrane surfaces, especially from abscesses, furuncles, and pustules. Also, the organism is found causing septicemia, meningitis, pneumonitis, and osteomyelitis. Recently it has been reported to be an important agent of a severe form of enterocolitis.

c. **Certain strains (phage types)** of *Staphylococcus* are notorious for their involvement in nosocomial infections. Newborn babies, post-surgical patients, and debilitating-disease patients are frequently infected.

d. **S. aureus** also is frequently the cause of *food poisoning outbreaks*, as the organisms grow very well in rich pastries, gravies, potato salads, and similar foods containing mayonnaise or salad dressing enriched with eggs. The poisoning is caused by those strains of the organism that produce a soluble, heat-stable enterotoxin. When contaminated food containing the organisms is improperly cooked, the organism may be killed, but the enterotoxin is not destroyed. After eating such foods, patients experience vomiting, diarrhea, and acute prostration within 2 to 6 hours. Affected individuals usually recover within 24 to 48 hours. The disease is rarely fatal. Staphylococcal food poisoning is an intoxication resulting from the ingestion of the enterotoxin. It is not an infection. Investigation should be conducted on suspect samples of foods ingested by the patient and not on stool specimens from such patients. More than likely, stool specimens from patients will not yield the causative organisms.

e. **Virulent staphylococci** produce several substances which account for their ability to invade tissue and cause disease. Among these are:

(1) Enterotoxin—a thermostable toxin which can resist boiling for a short period of time; it produces gastroenteritis in food poisoning.

(2) Coagulase—an enzyme that causes plasma to clot, thus creating a protective fibrin wall around a staphylococcal lesion.

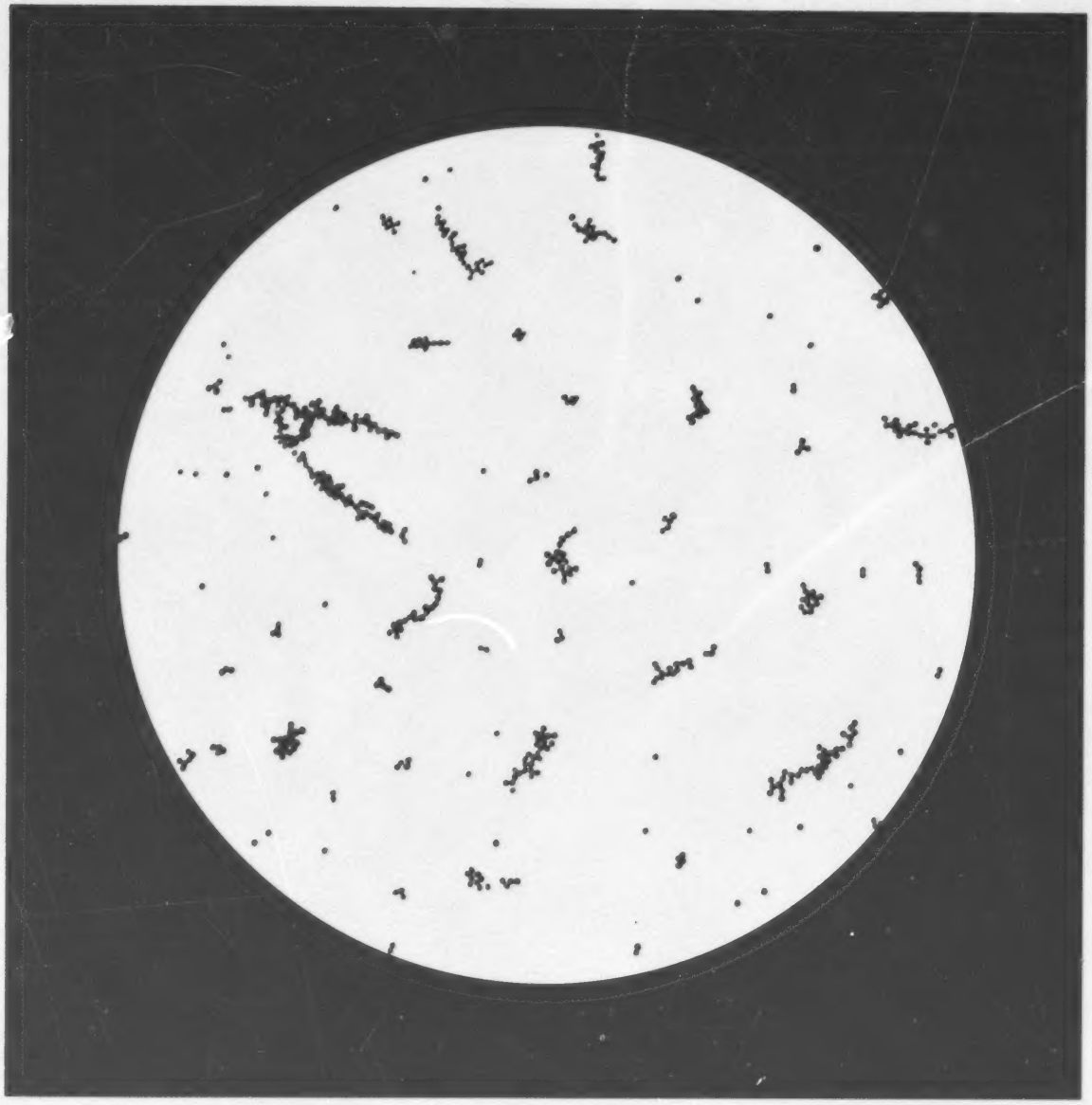

Figure 5–1. Gram Stain Smear of *Staphylococcus* sp.

(3) Leukocidin—a soluble substance which destroys white blood cells.

(4) Hyaluronidase—an enzyme active in the destruction of connective tissue.

(5) Staphylokinase (fibrinolysin)—an enzyme which dissolves fibrin clots, thus increasing the invasiveness of virulent organisms.

f. **S. aureus** frequently becomes resistant to treatment by most antimicrobial agents. This is especially true of those strains involved in nosocomial infections. In some instances, resistance to drugs is an inheritable characteristic and can be passed to successive generations of virulent organisms.

5–4. Growth Requirements:

a. Staphylococci are facultative anaerobes and grow well either aerobically or anaerobically. Micrococci are aerobes. Sarcinae and peptococci include both strict and obligate anaerobes.

b. Growth of the organisms can be obtained

on most simple nutrient media; on enriched media, such as blood agar, the organisms grow very well.

c. Aerobically, the staphylococci and micrococci grow better in an increased carbon dioxide atmosphere (5 to 10 percent CO_2).

d. The staphylococci are halophilic; they can grow on media containing 7 to 10 percent (w/v) sodium chloride. This characteristic is useful in differentiating and isolating staphylococci from related and similar-appearing organisms.

e. *S. aureus* can utilize (ferment) mannitol. Mannitol salt agar has been devised to selectively isolate *S. aureus* and as an aid in identifying the organism. Results are positive when the organism grows on the high salt content/mannitol media, producing yellow halos around the colonies. Since delayed fermentation of mannitol may occur with some strains, negative plates should be held an additional 24 to 48 hours before a final reading is made. *S. epidermidis* does not utilize mannitol and does not produce the yellow halo. This is a negative reaction. The yellow color is brought about by acids produced in mannitol utilization, which change the color of a phenol red indicator in the medium.

f. The single most important diagnostic characteristic for identification of the staphylococci is their ability to ferment glucose (anaerobically). The test should be performed in Baird-Parker medium with a vaspar seal. Hugh and Leifson's OF medium can be used to determine the mode of metabolism of glucose. Fermentative cultures can be placed in the genus Staphylococcus, and oxidative cultures in the genus Micrococcus.

5–5. Cultural Characteristics:

a. *S. aureus*, like many other pathogens, produces hemolysins capable of disrupting red blood cells. On blood agar, evidence of hemolysin production is seen as β hemolysis (clear halos) around growing colonies. On occasion, *S. epidermidis* also produces hemolysis.

b. Colonies are usually opaque, circular, smooth, raised, and 1 to 3 mm in diameter.

c. Colonies may or may not be pigmented. They can vary in color from white to golden yellow. Frequently, *S. aureus* is golden yellow when initially isolated. Such isolates must be further tested for coagulase production and mannitol fermentation, if necessary, to differentiate them from *S. epidermidis*.

d. The organisms grow best at 35°C.

5–6. Identification Procedures:

a. **DNase Test.** DNase medium is an excellent method for identifying colonies suspected of being *Staphylococcus aureus:*

(1) Inoculate the organism to the DNase medium using a wire loop.

(2) Incubate overnight to obtain growth.

(3) Following the incubation period, flood the plate with 1 N hydrochloric acid. A clear zone will appear around those colonies positive for DNase.

(4) As an alternate procedure, flood the plate with 0.1 percent toluidine blue. A rose pink color around the growth indicates that the colonies are positive for DNase production.

b. **Catalase Test:**

(1) Using an inoculating needle, select several good colonies from a plate which has been incubated 18 to 24 hours.

(2) Emulsify the colonies in a drop of 3.0 percent hydrogen peroxide.

(3) Rapidly effervescing bubbles indicate the presence of catalase. Care should be taken not to include blood cells from the media in the test, since they also will cause effervescence.

(4) All members of family *Micrococcaceae* are catalase-positive. Family *Streptococceae* are catalase-negative.

c. **Coagulase Test.** This test detects the presence of coagulase, a fibrin-clotting enzyme. There are two methods of performing the test. The slide coagulase test detects bound coagulase (clumping factor). The tube test, which is the more reliable of the two methods, detects free coagulase. By definition, *S. aureus* is always coagulase-positive; *S. epidermidis* is always coagulase-negative. The tests are performed as follows:

(1) *Slide Test:*

(a) Place a loopful of a centrifuged broth culture or a single colony picked from an agar plate on a slide.

(b) Mix it in a drop of 0.9 percent saline.

(c) Add a loopful of fresh human plasma, and mix the bacteria and plasma with the loop.

(d) If bound coagulase is present, clumps will form immediately.

(e) Run known positive and negative organisms as controls.

(f) To prevent variable results, use only *fresh* rabbit plasma (commercial product).

(2) *Tube Test:*

(a) Add either 0.1 ml of a broth culture or a colony from an agar plate to 0.5 ml of un-

diluted or a 1:4 dilution of rabbit plasma in a test tube; mix well.

(b) Incubate the tube in a 35°C waterbath.

(c) Examine at intervals of 39 minutes for 4 hours (staphylokinase may cause a false negative if interval checks are not made).

(d) Run known positive and negative controls.

(e) Any clot (loose or solid) is a positive test for *S. aureus*. Positives must be confirmed by gram stain because some gram-negative organisms give a positive test. Most positives occur within 1 hour.

d. **Examination of Cultures.** Typical colonies of staphylococci are generally formed on blood agar after 18–24 hours of incubation at 35°C. The presence of increased carbon dioxide in the atmosphere favors the development of typical growth on primary isolation. Mannitol-salt agar plates may be inoculated along with blood agar when examining specimens containing mixed flora, specifically for staphylococci. The high salt concentration of this medium will inhibit most organisms but not the staphylococci. Mannitol is also incorporated in the medium to enable detection of mannitol-positive strains which usually ferment this carbohydrate to acids. Pathogenic staphylococci appear as small colonies surrounded by a yellow zone, due to the action of such acids on phenol red indicator in the medium. Mannitol-negative (non-pathogenic) forms yield colonies surrounded by distinct red or purple zones on Bacto-mannitol-salt agar.

e. **Other Biochemical Tests.** Several other tests are sometimes run to confirm generic identification. These include nitrate reduction and gelatin liquefaction. Also, tests for phosphatase and deoxyribonuclease are sometimes performed because they correlate well with coagulase production. Except in unusual instances, these tests are not necessary for identification of staphylococci. Usually, the coagulase test, mannitol fermentation/DNase, and gram stain are all that is necessary.

5–7. Key for Identification. The key shown in table 5–1 is provided to aid in differentiation of aerobic members of family *Micrococcaceae*.

SECTION B—STREPTOCOCCI

5–8. Classification and Morphology:

a. **Taxonomy:**

Family II—Streptococcaceae
Genus I—*Streptococcus*
Family III—Peptococcaceae
Genus II—*Peptostreptococcus*

b. **Morphology:**

(1) All members of family Streptococcaceae are gram-positive and catalase-negative. Cells are nonmotile, nonsporulating cocci, 0.5 to 1.0 μm in diameter. Cells are spherical or oval.

(2) One species, *Streptococcus* (*Diplococcus*) *pneumoniae* is characteristically lancet-shaped. This organism is discussed separately in section C of this chapter.

(3) Streptococci are typically found arranged in chains; however, any of the species can be seen as single cells or as pairs of cells. For example, *S. pneumoniae* typically is seen in pairs (diplococci). However, this species will also be found arranged in chains if grown in serum-rich broth or if observed directly on a stained smear of cerebrospinal or pleural fluid (see figure 5–2).

c. **Classification Systems:**

(1) *Lancefield Classification.* This system is based on the antigenic properties of various types of streptococci. Precipitin reactions for a

Table 5–1. Differentiation of the Micrococcaceae.

Species	Gram Stain	Catalase	Coagulase	Mannitol	Anaerobic Glucose Fermentation	Nitrate Reduction	Phosphatase	Gelatin Liquefaction
	+	+	+	+*	+	+	+	+
	+	+	–	–*	+	+	+	–
	+	+	–	+	–	–	–	–
	+	+	–	+	–	+	–	–
	+	+	–	+	–	+	–	+

+ = Positive
– = Negative

* Most strains of [illegible] are mannitol positive.
Most strains of [illegible] are mannitol negative.

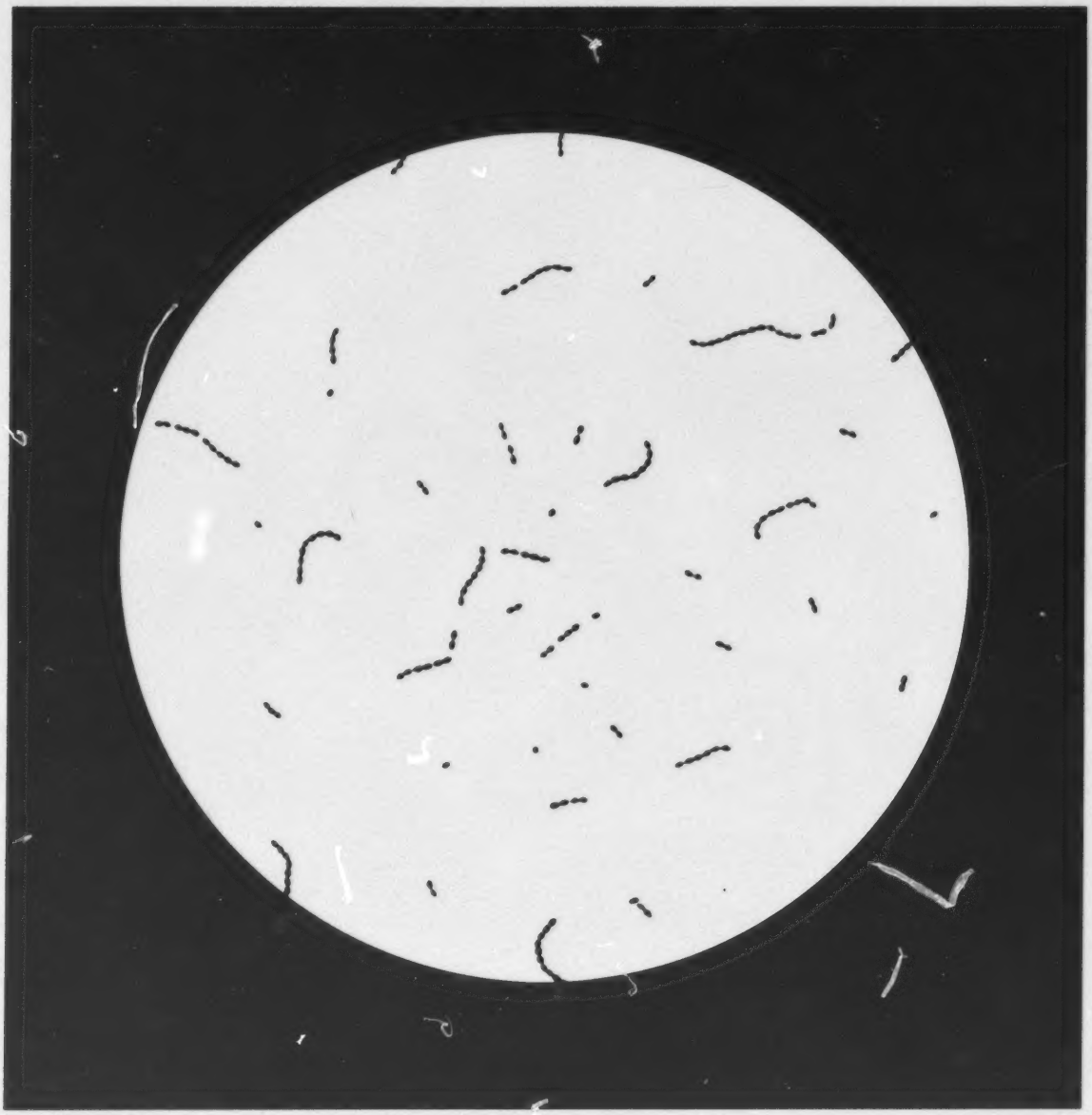

Figure 5–2. Gram Stain Smear of *Streptococcus* sp.

carbohydrate antigen (C substance) are different for each group in this series. The groups are designated by the letters A through H and K through T. Groups A, B, C, and G contain most of the streptococci that are considered to be human pathogens.

(2) *Sherman Classification.* A second classification, devised by Sherman, has as its basis both the physiologic and immunologic characteristics of the streptococci. The Sherman classification separates the streptococci into four groups with most human pathogens being in the first group.

(a) Pyogenes Group.
(b) Viridans Group.
(c) Enterococcus Group.
(d) Lactic Group.

(3) *Classification by Hemolysis.* For clinical purposes, the simplest classification of the streptococci is based upon the type of hemolytic reaction seen when the organisms are grown on sheep blood agar. These hemolytic

reactions are: alpha (green hemolysis), beta (clear hemolysis), or gamma (no hemolysis). Most human pathogens are beta hemolytic (frequently written β-hemolytic). The three classification systems are compared in table 5-2.

Table 5–2. Classification of Streptococci by Hemolysis.

[illegible]	[illegible]	[illegible]
Alpha	Not grouped	Viridans
Beta	A thru T	Pyogenes
Gamma	D (sp. faecalis)	Enterococcus

5–9. Pathogenicity:

a. *Streptococcus pyogenes* (Lancefield Group A) β-hemolytic) causes septic sore throat (streptococcal pharyngitis), scarlet fever, erysipelas, impetigo, acute bacterial endocarditis, and several other diseases. Septic sore throat is by far the most common of the diseases caused by this organism. Sometimes, 2 to 3 weeks following recovery from a β-streptococcal infection, patients develop acute glomerulonephritis or rheumatic fever. These conditions are not caused by a general bacteremia, but rather by the patient's hypersensitiveness to the original infection.

b. Group B streptococci are often involved in puerperal fever, other infections following childbirth, and meningitis in newborn babies.

c. Correct differentiation of group A streptococci from streptococci of other groups is important because of differences in drug sensitivity. Group A organisms are susceptible to penicillin while groups B, C, and D pathogens are frequently resistant to the antibiotic.

d. Group A streptococci produce several extracellular enzymes and other substances that increase their invasiveness. Among these are:

(1) *Streptokinase* (*fibrinolysin*). An enzyme capable of converting serum plasminogen to plasmin. Plasmin is an active proteolytic enzyme; it dissolves fibrin clots formed by the body in an attempt to wall off and isolate the infection site.

(2) *Streptodornase* (*desoxyribonuclease*). An enzyme which reacts with and reduces the viscosity of purulent exudates, permitting the spread of pathogens into tissue. The viscosity of such exudates is due primarily to desoxyribonucleoprotein, a substance which is liquefied by desoxyribonuclease.

(3) *Hyaluronidase.* An enzyme capable of denaturing hyaluronic acid, a principal constituent of the connective tissue holding cells together. This enables the pathogen to penetrate and spread throughout the tissue.

(4) *M Protein Antigen.* A type-specific substance whose presence is thought to correlate with virulence. It is found in group A streptococci that produce matt or mucoid colonies.

(5) *Erythrogenic Toxin* (*Dick Test*). The erythrogenic toxin is one which resists heat of 60 C for several hours, is soluble, and can be destroyed by boiling for 1 hour. It is responsible for the rash associated with scarlet fever. Production of the erythrogenic toxin is by the lysogenic streptococci. Susceptibility to the toxin can be demonstrated by the *Dick Test:* 0.1 ml of standardized diluted toxin (broth culture filtrate) is injected intradermally. A heat-inactivated sample is used as the control. The appearance in 8 to 24 hours of erythema measuring more than 10 mm in diameter is considered positive. (This test is very rarely used.) The Schultz-Charlton reaction is used to demonstrate the nature of the rash in scarlet fever—again, an antitoxin (specific in this case) is injected into the rash area. If the rash is caused by the erythrogenic toxin, the rash redness will become pale and fade near the point of injection. The antitoxin will have neutralized the toxin.

(6) Hemolysins. Substances excreted by streptococci that enable them to lyse red blood cells. Their presence is seen as alpha and beta hemolysis when the organisms are grown on sheep blood agar.

(a) The alpha hemolytic streptococci are normal inhabitants of the upper respiratory and intestinal tracts. They produce a greenish hue around their colonies. The green color is due to unidentified, reduced hemoglobin components. α-strep can cause disease in individuals with lowered physical resistance. α-streps have been implicated in subacute endocarditis, meningitis, and urinary tract infections.

(b) Beta hemolytic streptococci, other than group A, may be found as normal flora in respiratory tracts of up to 15 percent of the population. They produce a clear cell-free zone around the colonies, giving a clear yellowish hue to the media. Pathogenic implications are discussed in paragraph 5–9a.

(c) The nonhemolytic streptococci are practically all saprophytic strains found in various dairy products. Some strains have been

implicated in cases of subacute bacterial endocarditis. The nonhemolytic anaerobic streptococci (*Peptostreptococci*) capable of giving rise to suppurative lesions are found in the mouth, the normal vaginal flora, and the intestines. They usually have a putrid odor with accompanying pus cells.

(7) *Streptolysin S.* A nonantigenic hemolysin which is active hemolytically in the presence or absence of oxygen. Since S-hemolysin is not sensitive to atmospheric oxygen, it produces β-hemolytic reactions around surface colonies grown on blood agar.

(8) *Streptolysin O.* An antigenic hemolysin. Its production stimulates the body to produce antistreptolysin O antibodies, which are able to counteract its effects. An antistreptolysin O test has been devised to measure its production and thereby indirectly measure the severity of group A streptococcal infections. An antistreptolysin O test result of 250 Todd units or more is indicative of a recent streptococcal infection. The hemolysin is responsible for hemolytic reactions around colonies grown in the absence of oxygen. In the presence of oxygen the substance is nonhemolytic.

5–10. Growth Requirements:

a. A medium enriched with blood, ascitic fluid, tissue extracts, or similar substances is essential for growth of streptococci. Unenriched meat-infusion media, such as nutrient agar or broth, do not generally satisfactorily support the growth of *Streptococcus* spp. The standard blood agars and such broth media as thioglycollate, brain-heart infusion, Todd-Hewitt, and casein digest are successfully used in cultivating most species.

b. The majority of strains are facultative anaerobes. However, some strains are obligate anaerobes, and some are microaerophils that grow only in reduced atmospheric oxygen.

c. Growth is best at 35°C in an atmosphere of 90 percent N_2 and 10 percent CO_2. This insures the recovery of strains that do not tolerate atmospheric oxygen.

d. Streptococci should be cultured only on sheep blood agar. This will prevent confusing streptococci with *Haemophilus haemolyticus*, which also produces hemolysis but only on human or rabbit blood agar. *H. haemolyticus* is gram-negative and will not grow in media that do not contain human or rabbit blood.

e. Sodium azide is used in media prepared to selectively isolate streptococci, especially enterococci (group D, *S. faecalis*).

5–11. Culture Characteristics:

a. On blood agar, after 24 hours incubation, the streptococci generally appear as small (approximately 1 mm or less), slightly granular, circular, convex, translucent colonies.

b. Some groups of streptococci produce matt or mucoid colonies on primary isolation. These colonies possess M protein antigen. The M antigen is useful in the typing of group A streptococci. Types are numbered 1 through 50, and certain types correlate with virulence.

c. After repeated transfers on culture media, rough and smooth (R and S) colonies develop. These organisms are avirulent and do not possess M antigen. The antigen and virulence can be restored to the organism by repeated rapid animal passage.

5–12. Laboratory Identification of Streptococci:

a. **Direct Examination of Speciments.** Gram-stained smears of throat and nasopharyngeal materials and sputum are of limited value since saprohytic streptococci are usually present. Smears of spinal fluid are of definite value in revealing Gram-positive cocci predominant in chains. Smears of urinary sediments or exudates from localized lesions, wounds, or deep abscesses (peritonitis) may enable intelligent selection of culture procedure.

b. **Examination of Cultures.** The streptococci are such a heterogeneous group of organisms that neither morphological, physiological, nor immunological studies alone have been completely successful in classification of all species encountered. Hemolytic reaction is the criterion upon which streptococci are classified and reported. Clinical evidence coupled with the savings in resources supports the hemolytic criterion as being both practical and acceptable. The type of hemolysis exhibited on blood agar (alpha, beta, or gamma) affords tentative separation of streptococcal species. In all cases, suspected colony types should be verified as being streptococci by Gram-staining. The catalase test is useful in differentiating culturally similar beta streptococci and hemolytic staphylococcal forms. Staphylococci are catalase-positive while streptococci are catalase-negative.

c. **Identification of Beta Streptococci.** The beta streptococci are identified on the basis of cultural and physiological characteristics. In table 5–3, some significant biochemical reactions are listed which will enable identification of those groups responsible for the majority of human infections. It will be noted that the reactions obtained from *Streptococci faecalis*

(SF) and sodium hippurate broths do not separate group A from group C (human) streptococci. Such differentiation is not usually necessary since infections from members of each group generally respond to treatment with antibiotics and sulfonamides. With the exception of group D (enterococci), beta streptococci are sensitive to these agents. When dealing with a beta *Streptococcus* species, a most important laboratory procedure is that of determining whether the organism is an enterococcus (group D). This is successfully accomplished using SF broth. SF broth/Esculin broth is selective in that only *Streptococcus faecalis* and other members of group D streptococci are able to grow in it. The concentration of sodium azide in this medium will prevent the growth of other bacteria. The suspected pathogenic *Streptococcus* sp should be inoculated to SF broth from a pure colony. After 24-hours incubation at 35°C, the broth is examined for the presence or absence of growth. Visible growth is observed coupled with an indicator change from purple to yellowish brown. Sodium hippurate broth is used to enable differentiation of group B streptococci from groups A, C, and D. The medium is inoculated with the unknown and incubated for 48 hours. The broth is then tested for the possible presence of benzoic acid formed by the organism's hydrolysis of sodium hippurate. Optional procedures for the identification of beta streptococci are FA technique (immunofluorescence) and the Lancefield precipitin test.

d. **Identification of Gamma and Alpha Streptococci.** At present gamma and alpha streptococci, other than enterococci, can only be differentiated on the basis of colonial characteristics and reactions obtained from biochemical media. Table 5–4 may be consulted for simple identification of the well-recognized species (including enterococci). From a practical standpoint, it is only necessary to differentiate *Streptococcus faecalis* from other gamma and alpha streptococci. This is necessary because *Streptococcus faecalis* is resistant to penicillin, streptomycin, and sulfonamides, while the other gamma and alpha strains are sensitive to these therapeutic agents. It is also important to differentiate *Streptococcus* (*Diplococcus*) *pneumoniae* from alpha streptococci. Since pneumococcal strains form green hemolytic colonies similar to colonies of alpha streptococci on blood agar, it is necessary to employ the bile solubility test and other studies for their distinction. These procedures are discussed in the section dealing with pneumococci and are summarized in table 5–4.

e. **Identification of Anaerobic and Microaerophilic Streptococci.** At present, these organisms can only be identified on the basis of oxygen requirements and appearance on blood agar. A *Streptococcus* sp may be designated as anaerobic if it consistently grows only in the absence of free oxygen. Those streptococcal strains which grow poorly aerobically, yet form appreciable growth in the absence of free oxygen, are reported as microaerophilic streptococci. Both anaerobic and microaerophilic forms may exhibit alpha, beta, or gamma hemolysis.

SECTION C—PNEUMOCOCCI

5–13. Classification and Morphology:

a. **Taxonomy:**

Part 14.
Family II—Streptococcaceae
Genus I—*Streptococcus* (*Diplococcus*)
Species—*pneumoniae*

b. **Morphology:**

(1) The typical pneumococci are about 0.5 µm in diameter, gram-positive, nonmotile, encapsulated, and occur in pairs. Lancet shapes on the ends with adjoining sides flattened or slightly curved are often seen in stained preparations (see figure 5–3).

(2) Stained sputum or pus smears often show single cocci or chains. The organism also stains gram-negative and becomes self-lytic with aging.

5–14. Pathogenicity:

a. About 80 percent of the instances of pneumonia in adults are caused by pneumococci types I thru VIII. More than half of all fatal bacteremia in the same group are attributed to the same types. In cases where children are involved, type XIV is frequently isolated.

b. The pneumococci produce disease by using their ability to multiply well in the tissues. They produce no toxins, but produce instead capsules composed of polysaccharides which impede or prevent their engulfment (phagocytosis) by protective cells in the body.

c. Infection with pneumococci causes outpouring of fibrinous edematous fluid into the alveoli followed by erythrocytes and leukocytes, which clump in areas of the lung.

d. The pathogenic pneumococcus does not confine itself to the lungs; it can be found in the throat, saliva, and secretions from the upper respiratory tracts of individual carriers. During various seasons of the year, pneumo-

Table 5–3. Biochemical Reaction of Streptococci.

Species	Lancefield Group	Hemolysis	Growth in 5-7% NaCl	Growth 45C	Growth pH 9.2	Hippurate Hydrolysis	Aesculin Hydrolysis	Arginine Hydrolysis	Acid Production in Carbohydrates			
									Arabinose	Maltose	Trehalose	Glycerol
S. pyogenes	A	β	–	–	–	–	–	+	–	+	+	–
S. agalactiae	B	α, β	–	–	–	+	–	+	–	+	+	+
S. dysgalactiae	C	α	–	–	–	V	–	+	–	+	+	–
S. equisimilis	C	β	–	–	–	–	V	+	–	+	+	+
S. equi	C	β	–	–	–	–	–	+	–	+	–	–
S. zooepidemicus	C	β	–	–	–	–	+	+	–	V	–	–
S. bovis	D	α, γ	–	+	–	–	+	–	V	+	V	–
S. equinus	D	α	–	+	–	–	+	–	–	+	–	–
S. faecalis	D	β, γ	+	V	V	V	+	+	–	+	V	+
S. faecium	D	β, γ	+	+	+	V	+	+	–	+	+	+
S. durans	D	α, β	+	+	+	V	+	+	+	+	+	+
S. sanguis	H	α	–	V	–	–	+	+	–	+	+	–
S. hominis	K	γ	–	+	–	–	+	–	–	+	+	–
S. lactis	N	α	–	–	–	V	V	+	V	+	V	–
S. pneumoniae	N	α	–	–	–	–	V	–	–	–	V	–
S. mitis		α	–	V	–	–	–	V	–	+	–	–
S. uberis		α	–	+	–	+	+	+	–	+	+	+
S. pneumoniae		α	–	–	–	–			–	+	+	+

\+ = 80-100% Positive
– = 0-20% Negative
V = Variable

α = alpha
β = beta
γ = gamma

Table 5–4. Differentiation of Pneumococcal Strain From Alpha Streptococci.

Organism	Hemolysis	Colonial morphology	Capsules	Swelling Reaction in Type-Specific Antiserum	Solubility in Sodium Desoxycholate	Optochin Sensitivity
Pneumococci	Alpha	Small, flat, shiny or mucoid colonies with concentric rings and depressed centers.	Present	Reactive	Soluble	Sensitive
Alpha Streptococci	Alpha	Small, raised, dome-shaped, smooth colonies which are translucent or opaque.	Absent	Nonreactive	Insoluble	Insensitive

NOTE: Colonies of pneumococcus type III are generally larger, raised, and more mucoid, with a tendency to form confluent growth. These forms resemble droplets of oil.

cocci can be isolated from the pharynx of up to 7 percent of normal humans tested by culture. Pneumococci also can be recovered from sputum, blood, and exudates of persons suffering from lobar pneumonia, and from cases of mastoiditis, meningitis, peritonitis, endocarditis, and otitis.

5–15. Growth Requirements. Generally, the pneumococcus is a difficult organism to culture due to its complex nutritional requirements. It has been found however, that for routine laboratory work, trypticase soy broth or trypticase soy agar is a satisfactory medium for pneumococcal cultures. Pneumococci can be grown aerobically, but in that they lack cytochrome granules, they are biochemically close to anaerobes. Pneumococci will grow well on trypticase soy agar containing defibrinated blood because catalase provided by the blood aids the organisms in neutralizing the hydrogen peroxide produced. They will not thrive in media containing glucose due to the metabolism of the glucose to lactic acid producing a lowered pH of the media (optimum pH 7.6). The pH change is toxic to pneumococci need for CO_2 varies in about 5 to 10 percent of the existing strains of the organism. Their requirement for CO_2 is higher than atmospheric concentration. Therefore, cultures should be routinely incubated in a CO_2 incubator or candle jar to insure recovery of the organisms. Incubation should be at 35°C for 18 to 24 hours. Pneumococci grow very well in blood agar base, containing sheep's blood and meat-infusion broth incubated under 10 percent carbon dioxide conditions.

5–16. Cultural Characteristics:

a. When grown on trypticase soy agar containing 5 percent blood under increased carbon dioxide, the pneumococci form small round colonies, which are about 1 mm in diameter, surrounded by a zone of alpha hemolysis. Anaerobically grown pneumococci, using the same medium, exhibit beta-hemolysis with young colonies that appear dome-shaped but later sink in their center, due to cellular autolysis ("Phantom Colonies"). Colonies may give the identical appearance of an alpha-streptococci (young or compact colonies) and therefore be overlooked. The typical pneumococcal organism isolated from the human host is encapsulated.

b. Pneumococci will grow very well in thioglycollate medium. No pellicle is formed as with the *Bacillus* sp, but in older cultures the organisms may settle to the bottom of the tube.

c. Liquid media, such as trypticase soy broth, is excellent for the growth of the pneumococci and can be enriched with the addition of glucose to a final 1 percent concentration. In this instance, however, a neutralizer must be added periodically to counteract the lactic acid formation. Alkalizing agents, such as sodium hydroxide, sodium carbonate, sodium bicarbonate, or calcium carbonate, may be used to adjust the pH to 7.6. The addition of a few drops of an indicator, such as phenol red (alcoholic solution), is an easy method of determining when the solution is more on the acid side.

5–17. Laboratory Identification of Pneumococci:

a. **Direct Examination of Specimens.** Gram-stained smears of sputum specimens may reveal the typical Gram-positive, lancet-shaped diplococci. The organisms also may be observed

Figure 5-3. Hiss Capsule Stain of *Streptococcus Pneumonia* From Initial Isolation in Broth Culture.

in spinal fluid sediments, pleural fluids, or other exudates from infected tissue. The presence of capsules may be noted in Gram-stained smears as thick halos around the cells when observed under reduced light. Demonstration of the capsule is better accomplished by preparing a capsule stain or an India ink preparation as described in chapter 3. Pneumococci may be quickly and accurately identified by serologic typing. If Gram stains of clinical materials yield possible pneumococci in relatively pure form, samples of specimens may be reacted with type-specific antisera on a glass slide and observed for capsular swelling (Quellung reaction). If capsular swelling is observed in any one of the antiserum pools, additional films may be prepared and tested with the individual typing serums making up the particular

serum pool wherein a positive reaction occurred. Specific capsular swelling will occur in the homologous typing serum (table 5–5).

Table 5–5. Pneumococcal Typing Antiserum Pools.

b. **Examination of Cultures.** On blood agar plates pneumococcal colonies are generally well formed after 18 to 24 hours incubation in a candle jar at 35° C. Plates should not be discarded before 48 hours incubation time since primary growth of certain types develop slowly. Colonial growth only may be obtained on blood agar plates streaked from primary broth cultures. Although typical colonies of pneumococci are rather distinctive, the growth cannot be grossly distinguished with certainty from that of alpha hemolytic streptococci. No report should be rendered as to the presence of pneumococci or alpha streptococci without performing confirmatory studies, summarized in table 5–4.

c. **Confirmatory Procedures:**

(1) Isolated colonies suggestive of pneumococcus are examined as follows:

(a) Prepare Gram-stained smears and examine for Gram-positive diplococci which may be lancet-shaped.

(b) Prepare a capsule stain or India ink mount and observe for evidence of capsules (chapter 3).

(c) Perform slide agglutination tests and observe for capsular swelling (Quellung reaction).

(d) Perform the sodium desoxycholate (or bile) solubility test and observe for dissolution of growth.

(e) Inoculate a blood agar plate for determination of optochin sensitivity. The optochin sensitivity test may be performed in lieu of solubility tests. Optochin (ethylhydrocupreine hydrochloride) discs are placed on the heavily inoculated plate. After 18 to 24 hours incubation pneumococcal growth will exhibit a large clear zone of inhibition.

(2) The results obtained with the above procedures along with gross culture examination will enable differentiation of pneumococci from alpha streptococci, as shown in table 5–4. Since type-specific pneumococcal antisera are not available to most laboratories, serological identification must frequently be omitted. The results of the desoxycholate solubility test along with careful colonial and microscopic studies are usually sufficient criteria for identification of pneumococci. The mouse virulence test described below may be helpful in determining whether or not a given strain is pathogenic. This procedure affords rapid identification of virulent pneumococci, especially when specimens are inoculated directly.

d. A summary of the procedures or tests used in the identification of *S. pneumoniae* includes Quellung reaction, optochin sensitivity, bile solubility, and insulin fermentation.

(1) *Quellung Reaction.* The Quellung reaction is the simplest, quickest, and most rapid method of identifying the pneumococcus, but it is seldom used today. At one time the treatment of pneumonia was based on whether or not the organism was encapsulated. Pneumonia is no longer treated using such guidelines. The organisms were mixed with a specific antiserum which caused the capsular substance surrounding them to expand or swell. A small amount of methylene-blue stain was added to give color to the organism, making it more visible.

(2) *Optochin Sensitivity.* This test is widely used in the detection and identification of *S. pneumoniae.* It is especially helpful in the detection of the pneumococcus in a mixed culture. Throat cultures are screened for the organism by first streaking the plate with the culture swab, then applying an optochin disc in the central portion of the streak. If the culture is positive, a zone of inhibition will appear in the area containing *S. pneumoniae.* Optochin disks are impregnated with a 1:4000 solution of ethylhydrocupreine hydrochloride and dried prior to use (available commercially).

(3) *Bile Solubility.* Testing by solubility detection is accomplished by plating a few drops of 10 percent sodium desoxycholate or sodium taurocholate to a fresh broth culture, buffered to a pH of 7.4. The haze will disappear from the solution in 5 to 10 minutes. The test is considered positive. Occasionally strains of alpha hemolytic or nonhemolytic streptococci will give a positive test; therefore, the test should not be used alone as a test for the identification of pneumococci.

(4) *Inulin Fermentation.* Inulin is fermented by *S. pneumoniae,* producing acid and

no gas. It is fermented by most strains of the organism, and also by *S. salivarius* and *S. sanguis*.

5–18. **Key for Identification.** Table 5–4 shows differentiations between pneumococci and alpha streptococci.

Chapter 6

GRAM-NEGATIVE COCCI

6–1. Classification and Morphology:

a. **Taxonomy:**

Part 10.
Family I—Neisseriaceae
Genus I—*Neisseria*
Part 14.
Family II—Streptococcaceae
Genus V—*Gemella*
Part 11.
Family I—Veillonellaceae
Genus I—*Veillonella*

b. **Morphology:**

(1) All of the members of the family *Neisseriaceae* are morphologically similar in that they all are Gram-negative, coffee-bean-shaped diplococci with flattened adjacent sides and an average diameter of 0.5 to 1.0 μm (figure 6–1). They are nonmotile and nonsporeforming. Some authorities say that the cells of *N. meningitidis* have slightly flattened surfaces on adjacent sides while those of *N. gonorrhoeae* are slightly cupped or concave.

(2) The Gram stain reaction is clear-cut. However, in some smears, particularly thick smears and those made directly from purulent specimens, the organisms may resist decolorization with 90 percent ethanol, used alone. Ether-alcohol (50 percent) or acetone (100 percent) gives more consistent decolorization. Known Gram-positive and Gram-negative controls should be stained at the same time to monitor the procedure.

(3) *Neisseria meningitidis* and *Neisseria gonorrhoeae* are generally considered to be the only virulent pathogens in the family Neisseriaceae. Although these two are encountered more frequently than other species of the genera, saprophytic types are not excluded from possible involvement. The two more common species are discussed here in more detail.

6–2. *Neisseria meningitidis*:

a. **Pathogenicity.** *N. meningitidis* is the cause of epidemic meningococcal meningitis, meningococcemia (septicemia), and bacterial endocarditis. The organism has been found to cause disease only in humans and sometimes causes outbreaks resulting in many fatalities. Meningococci are frequently isolated from normal mouth and nasopharyngeal specimens. Sometimes they are found in urethral and vaginal specimens, in which case they may be confused with expected gonococci. The normal carrier rate can run as high as 50 percent without an outbreak occurring. Some of the highly communicable strains of the "meningococcus" outbreaks are really a new serotype. They have been known to occur among troops confined to a small area and seem to have a peculiar predilection for the armed forces' recruit or basic training bases.

b. **Direct Gram Staining.** Direct Gram-stains should be made of all specimens taken in cases suspected of meningococcal infection. On smears the organism is frequently seen as an intracellular inclusion within a white blood cell. Cells are frequently capsulated. Also, the organism will sometimes be found in exudates of rashes and in blood smears taken from patients having meningococcemia. It also is a normal inhabitant of the nasopharynx. *Neisseria* cannot be presumptively identified as a pathogenic species in specimens from this area because of the presence of saprophytic Neisseria. Organisms isolated in suspected meningitis should be positively identified by the indophenol oxidase test and by inoculating colonies of a pure culture of the bacteria on fermentation sugars, glucose, maltose, and sucrose.

c. **Isolation Procedures:**

(1) Uncontaminated specimens, such as spinal fluid, blood, or fluid from a joint, should be cultured on Mueller Hinton, blood (or chocolate) agar, Thayer-Martin medium, and in brain-heart infusion broth or casein digest broth. Incubate cultures 18 to 24 hours at 35°C in a moist candle jar of CO_2 incubator. Increased humidity favors growth and good colony development.

(2) Contaminated specimens such as pus, exudates, and nasopharyngeal specimens collected in investigating carriers should be cultured on Thayer-Martin medium which is selective for *Neisseria* and nonsupportive for the other bacteria present. Addition of the antibiotics vancomycin, colistin, and nystatin makes the medium selective for most pathogenic *Neisseria* species. Prior to plating, the medium should be warmed to room temperature to prevent possible loss of the bacteria due to the lower temperature. Incubate cultures by placing them in moist CO_2 atmosphere at 35°C for 18 to 24 hours.

d. **Identification Procedures:**

(1) *Gram Stain.* Purulent specimens frequently show meningococci as intracellular in-

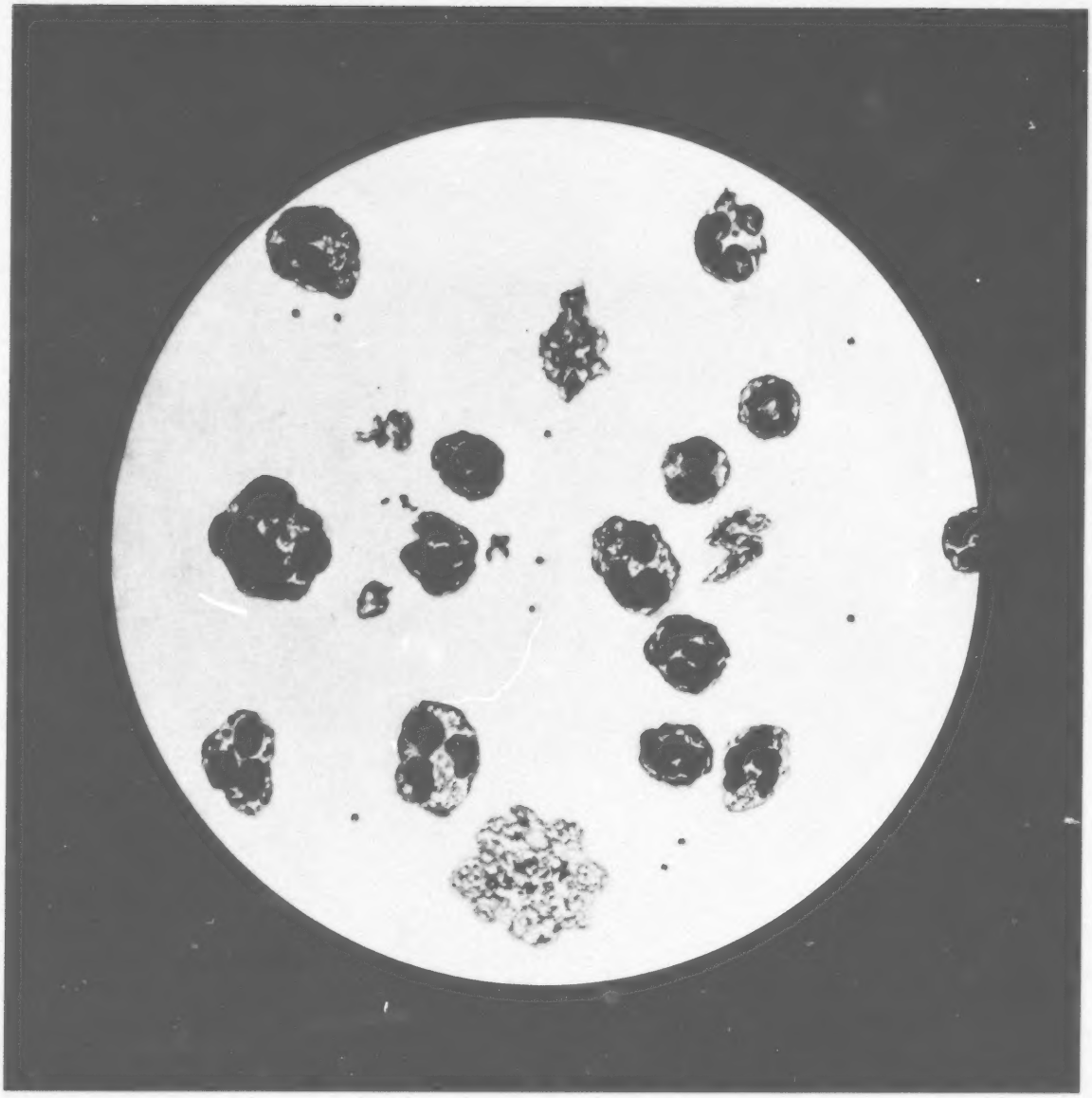

Figure 6–1. Gram-Stained Smear of *Neisseria Gonorrhoeae.*

clusions. The bacteria may enter only a small number of cells, thus requiring a diliger search of the stained specimen before any bacteria is observed.

(2) *Colony Morphology.* On blood and chocolate agar and on Thayer-Martin medium, colonies are smooth, glistening, translucent, and soft. No pigmentation is usually present; however, strains of serological groups A and C are irridescent (bluish-grey). Strains of group B occasionally become yellowishly pigmented, especially in old cultures. Autolytic changes rapidly produce swollen cells known as involution forms. Also, plates showing little or no growth should be examined with a stereo microscope because the meningococci can appear as very small L-form colonies, especially if the specimen is from a patient who has been treated with an antibiotic.

(3) *Oxidase Test.* Add a loopful of aqueous oxidase reagent (1 percent tetramethyl-p-phenylenediamine dihydrochloride, or 1 percent N, N-dimethyl-p-phenylenediamine monohydrochloride, prepared fresh) to a selected

colony. If the colony is *Neisseria*, it will quickly turn pink, then black, indicating the presence of cytochrome oxidase. Alternately, the oxidase test can be performed using the method of Kovacs (test for *Pseudomonas*). Smear a suspected colony on a strip of Whatman No. 1 filter paper previously moistened with the reagent. The smeared area should turn purple in 10 to 15 seconds. Only a platinum loop should be used here, because loops containing iron (Nichrome) give false positive tests. *Pseudomonas* and *Alcaligenes* also can give positive tests, but these organisms are usually differentiated by morphological differences on Gram staining. *Pseudomonas* can be detected by its sweet, fruitlike odor or the metallic sheen on blood agar or TSI.

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(4) *Carbohydrate (Sugar) Tests.* These tests are performed in tubes containing 2 ml of cystine-trypticase agar supplemented with sterile solutions of selected carbohydrates to a final concentration of 1 percent. Since the *Neisseriaceae* (except for *N. flavescens*) largely utilize sugars oxidatively, a heavy inoculum should be applied on the *surface of the agar*. Tubes are incubated at 35°C for 16 to 20 hours and are read immediately after the time specified. Delay in reading results can cause confusion and inconcistent results. These confusing results occur because sometimes only a small amount of acid is produced and the change in the indicator may be slight. Tubes showing no growth should be held for 5 days before discarding. The results of carbohydrate tests and other criteria are given in table 6–1.

(5) *Growth Temperature Tests.* The nonpathogenic *Neisseria* are usually able to grow at 22°C and at 35°C. *N. meningitidis* and the other pathogen, *N. gonorrhoeae*, will not grow at 22°C. For the test, two plates of chocolate agar are inoculated with a diluted suspension of the bacteria; one is incubated at 22°C and the other at 35°C.

(6) *Serological Tests.* If possible, isolates should be serologically grouped. Antisera to surface antigens are commercially available. The test is performed as a slide agglutination procedure. The antisera also can be used to perform a Quellung grouping reaction directly on the sediment of spinal fluid and other uncontaminated specimens. Serological groups A, C, and D will react. Group B seemingly contains no surface antigens and therefore will not react with the antisera. The antisera are available as a polyvalent grouping antisera and as a group specific antisera. Serological testing is important because some strains are highly virulent and cause epidemics while other strains are less virulent and are less often isolated in epidemics.

6–3. *Neisseria gonorrhoeae:*

a. **Pathogenicity:**

(1) The gonococci invade the mucous membranes of the genitourinary tract and cause gonorrhea. In males, gonorrhea is an acute anterior urethritis. The disease is usually accompanied by large amounts of purulent mucoid exudate and frequently an intense painful burning sensation. In females the disease is very much less acute and can be asymptomatic.

(2) Gonorrhea is a venereal disease and with few exceptions is transmitted by sexual intercourse. In untreated cases the disease does not stay confined to the genital areas. In males it can eventually spread to the prostate, epididymis, and rectum. In females, the fallopian tubes, uterus, ovaries, peritoneum, and rectum are frequently involved. In old untreated cases the disease can spread in both male and female to other parts of the body, such as joints, conjunctiva, heart valves, and meninges.

(3) An infected mother can transmit the disease to her newborn as it passes out of the uterus. The result is most often an infection of the eyes (gonorrheal ophthalmia). Initially the conjunctiva are infected, but the infection rapidly spreads and, if untreated, can result in permanent blindness. For this reason, the law requires that the eyes of all newborn babies be treated with silver nitrate solution, penicillin ointment, or other equivalent bacterial agent immediately after delivery.

(4) On occasion, gonorrhea is seen in epidemic outbreaks among small girls in close contact situations, such as orphanages, boarding schools, etc., and causes a vulvovaginitis in those afflicted.

(5) The disease occurs among homosexuals, and in many instances the primary sites of infection are the rectum and pharynx. Thorough examination is a must for detection of these cases.

(6) Man is the only known natural host for the disease. It will be found to occur in the young, the middle-aged, and the older person. Because of the changes in life style, it is now found more frequently in the preteen and young adult.

(7) Previously, many asymptomatic cases went undetected. Techniques are now available for diagnosing these cases. Therefore, all vaginal and urethral specimens submitted in con-

Table 6–1. Carbohydrate Criteria of *Neisseria* spp.

Species	Growth Characteristics	Growth at 22C	Acid Production in Carbohydrate					Growth on			
			Glucose	Sucrose	Maltose	Fructose	Lactose	Nutrient Agar	Thayer Martin	Oxidase	Catalase
[illegible]	No pigment. Convex, smooth, glistening, transparent, grey white colonies, 0.5 to 1.0 mm.	–	+	–	–	–	–	–	+	+	+
[illegible]	No pigment. Smooth, mucoid, round, translucent, bluish grey and yellowish colonies, 2-3 mm. Capsulated.	–	+	–	+	–	–	–	+	+	+
[illegible]	Opaque, grey white, friable colonies, difficult to emulsify.	+	–	–	–	–	–	+	–	+	+
[illegible]	Most strains pigmented; greenish yellow to yellow. Smooth, convex, and adherent.	+	+	±	+	±	–	+	–	+	+
[illegible]	Golden yellow pigment. Smooth, mucoid, glistening translucent colonies.	+	–	–	–	–	–	+	–	+	+
[illegible]	Light brown to caramel colors.	+	–	–	–	–	–	+	–	+	+
[illegible]	Resembles N. meningitidis. Some strains yellowish. Mucoid.	–	+	–	+	–	+	+	+	+	+
[illegible]	Large, round, convex greyish translucent, mucoid colonies, 3-4 mm. Capsulated.	+	+	–	–	+	–	+	–	+	+
[illegible]	Smooth, grey white. Grows slowly. Large zone of hemolysis.	+	+	+	+	+	–	+	–	–	–

*The genus *Neisseria* includes previous species: *N. catarrhalis*, *N. flava*, *N. sordida* and *N. subflava*.

+ = Positive reaction or growth.

– = Negative reaction or growth.

ditions such as nonspecific urethritis and common vaginitis should be routinely cultured on Thayer-Martin medium to detect and prevent further spread of the disease by asymptomatic carriers.

(8) Reports indicate that some strains of gonococci acquired in Southeast Asia do not produce the usual gonorrheal symptoms of discharge and pain in males. These individuals are reported to remain asymptomatic. The culture techniques given here are adequate for recovering the organism in these cases. Here, the specimen for culture is best collected on a sterile wire loop inserted into the orifice of the urethra. Prostatic massage may aid in the recovery of bacteria in persistent but difficult cases. The patient usually complains of difficulty but cannot collect a slide specimen adequate for diagnosis.

b. **Direct Gram Staining:**

(1) In cases of acute gonorrhea in males, demonstration of intracellular Gram-negative diplococci is *sufficient* for diagnosis. Prepare the smear by rolling the swab on the slide; do not rub because rubbing distorts cellular morphology.

(2) Specimens from males containing too few organisms to diagnose the disease from a direct smear and specimens that are found to be grossly contaminated should be cultured on Thayer-Martin medium. Isolation of oxidase-positive, Gram-negative diplococci is not sufficient evidence for a diagnosis of gonorrhea. Diagnosis should be based on the Gram stain, the oxidase test, and sugar fermentation rather than any single procedure.

(3) Direct Gram staining of specimens from the pharynx or anal canal is not recommended because of the presence of morphologically similar nonpathogenic species. These specimens should be processed by culturing on Thayer-Martin medium and confirmed by carbohydrate testing of oxidase positive isolates.

(4) Specimens from females should be

collected from the endocervical canal. Vaginal specimens should be collected only when endocervical specimens are not collectable or when the patient is a child. Diagnosis of gonorrhea in a female should be made only after the specimen has been cultured on Thayer-Martin medium and the isolates are found to be oxidase-positive, Gram-negative diplococci. Confirmation by carbohydrate testing is a must.

c. **Isolation Procedures:**

(1) *Male Urethral Specimens.* Collect the specimen by getting the patient to firmly strip the urethra toward the orifice. Collect the exudate that appears on a sterile loop or less preferably a cotton-tipped swab. Make a smear. If necessary, inoculate the specimen in thioglycollate broth, or blood agar and Thayer-Martin medium in the manner shown in figure 6–2.

(2) *Adult Female Specimens.* Specimens from adult females should be collected using a speculum moistened with water. Soap or any other disinfectant should not be used. Collect the exudate directly from the endocervical canal. A Gram-stained smear can be made and examined. Diagnosis of gonorrhea should not be made until oxidase-positive, Gram-negative diplococci, grown on Thayer-Martin medium and confirmed by carbohydrate tests, have been isolated.

(3) *Young Female Specimens.* When necessary, vaginal specimens should be obtained using a speculum and the specimen taken from the posterior vaginal vault. In young females having intact hymens, collect the specimen from the vaginal orifice with a sterile loop or cottontipped swab. Culture on Thayer-Martin medium. Isolates collected in medicolegal cases and those from children must be confirmed as *Neisseria gonorrhoeae* by carbohydrate testing.

(4) *Anal Specimens.* Anal specimens are

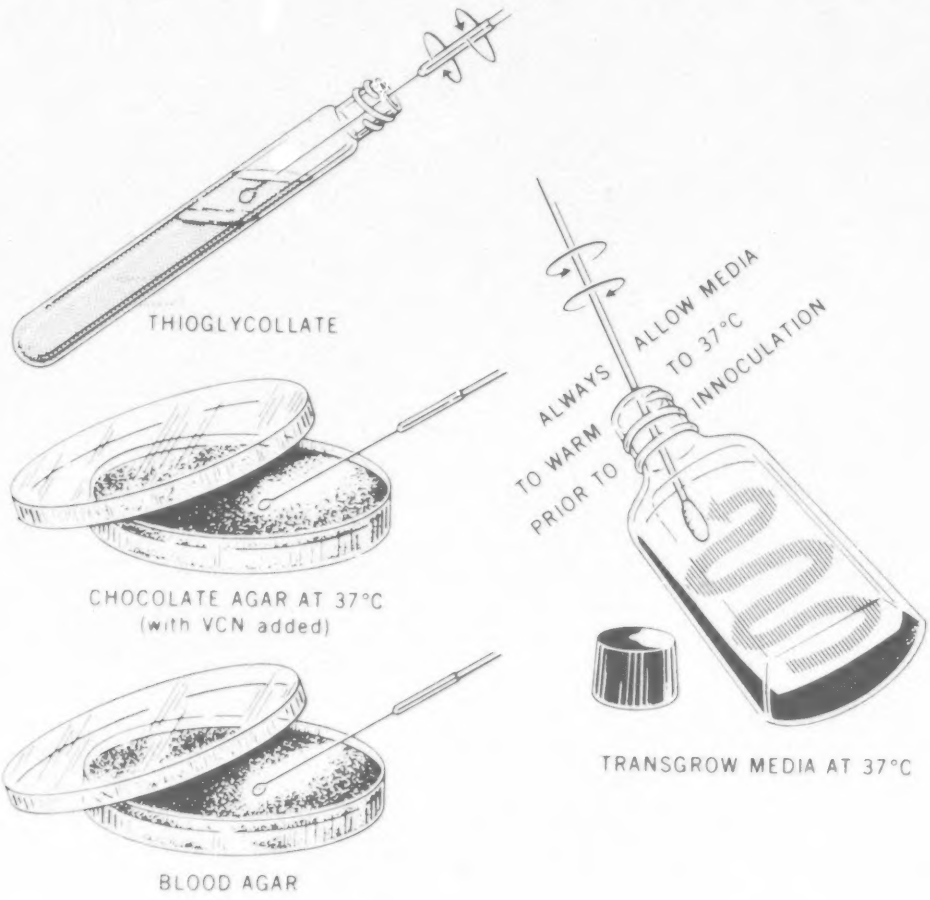


Figure 6–2. Inoculation of Media for *Neisseria gonorrhoeae.*

collected by inserting a cotton-tipped swab about 1 inch into the anal canal. Avoid contaminating the swab with feces; do not push in too deeply. If the swab gets contaminated with feces, use another swab. Culture on Thayer-Martin medium. If oxidase-positive organisms are isolated, confirm by Gram stain and carbohydrate testing.

(5) *Thayer-Martin Medium.* This medium is used for all specimens cultured in suspected gonorrhea. The medium is compsed of GC agar base, prepared as a chocolate agar supplemented with IsoVitaleX (BBL) or Supplement A, B, or C (Difco). Antibiotics (vancomycin, colistin, and nystatin) are added to inhibit Gram-negative, Gram-positive organisms and yeast that confuse the picture or overgrow the pathogen. Inoculate plates as shown in figure 6–2.

(6) *Blood Agar.* A blood agar plate and an enriched broth medium, such as casein digest broth or thioglycollate broth, should be inoculated along with the agar medium. This provides for the recovery of other organisms which may be involved (*E. coli, Proteus sp, S. aureus, S. faecalis,* etc).

(7) *Culture Inoculation.* Cultures are best inoculated in the clinic or on the ward at the time the specimen is collected. If immediate processing is not possible, inoculate the specimen on Transgrow medium (Difco), or place the swab sample in Stuart's transport medium or a culturette, containing a holding preservative. Culture specimens should not be subjected to cold temperatures, nor should there be undue delay before inoculation and incubation. *N. gonorrhoeae* is very susceptible to temperature change and therefore will survive only in a small temperature range.

(8) *Incubation.* Incubate cultures at 35° C (some *N. gonorrhoeae* do not grow well at 37° C) in a moist candle jar or CO_2 incubator. Moisture is important. Some of these organisms grow well without the CO_2, but few grow well without moisture. However, do not permit too much moisture because this favors the growth of spreaders.

d. **Identification Procedures:**

(1) *Gram Stain.* Gonococci are seen typically as inclusions in white blood cells. Cells also are seen extracellularly in pairs and in small clumps.

(2) *Colony Morphology.* On chocolate agar, colonies of *N. gonorrhoeae* are usually opaque, grayish-white, glistening, and convex. Colonies become mucoid after 48 hours of incubation. Colonies are 1 to 4 mm in diameter.

(3) *Oxidase Test.* See paragraph 6–2d(3).

(4) *Carbohydrate Tests. N. gonorrhoeae* inoculated to glucose, maltose, and sucrose ferments only glucose with production of acid without gas (some strains fail to ferment glucose). Use cystine, trypticase semisolid agar (BBL, CTA Medium, Difco, Cystine Tryptic Agar) as a basic medium, adding aseptically 1.0 percent sugar previously filter-sterilized. Carbohydrate reactions are given in table 6–1.

(5) *Growth Temperature Tests.* See paragraph 6–2d(5).

(6) *Fluorescent Antibody Staining (FA).* FA is not recommended for direct staining of urethral, vaginal, anal, or pharyngeal specimens. Gram staining will be sufficient when pus is abundant, allowing for presumptive diagnosis of gonorrhea. In a number of cases, however, especially those of female infections, gonococci are not found, or cannot be identified because numerous other bacteria are present. And when chronic infections cannot be diagnosed using the routine staining procedure, the FA technique is relied upon for positive identification. FA is of value in examining conjunctiva and joint fluid specimens. Also, FA is useful as a confirmatory tool when *N. gonorrhoeae* is grown on selective media.

6–4. Nonpathogenic Gram-Negative Diplococci:

a. **Morphology:**

(1) The saprophytic *Neisseria* species and *Gemella haemolysans* (formerly *N. haemolysans*) are morphologically similar to the meningococci and gonococci in size, shape, and staining characteristics. *G. haemolysans* has been found to stain Gram-indeterminate with Gram-positive type cell walls and has therefore been placed in the Family Micrococcaceae.

(2) Cells are found arranged singly, in pairs, in tetrads, and in small groups. Some species occasionally form dense clumps of cells.

(3) Some species (for example, *N. mucosa* produce capsulated cells similar to *N. meningitidis* on initial isolation.

(4) Uneven staining is frequently seen. The unevenness is thought to be due to the fact that the members of *Family Neisseriaceae* possess characteristics associated with pyogenic Gram-positive cocci, in spite of their Gram-negativeness. Consistent staining can be obtained by following the technique recommended in paragraph 6–1b(2).

(5) In old cultures, autolytic changes result in the production of involution forms, which are large swollen cells that stain poorly.

The pathogenic *Neisseria* also produce these forms but do it much more quickly.

b. **Pathogenicity:**

(1) The saprophytic Gram-negative diplococci are normal inhabitants of the nasopharynx and the female genital tract.

(2) Although commonly referred to as nonpathogenic, these organisms are parasites, are opportunistic, and sometimes cause disease. They have been reported in bacterial endocarditis, nonmeningococcal meningitis, tooth abscesses, conjunctivitis, and other diseases.

(3) Isolates from throat, sputum, and cerebrospinal fluid specimens are often confused with meningococci. Isolates from the female genital tract are confused with gonococci. Isolates in both instances should be positively identified by performing carbohydrate and growth temperature tests.

(4) Some taxonomists have combined *N. sicca*, *N. flava*, *N. perflava*, and *N. subflava* in a single species called *N. subflava*. The combination is made on the basis of similar antigenic properties in these species. The new nomenclature is used in paragraph 6–1 and elsewhere.

c. **Growth Requirements:**

(1) The nonpathogenic species, except for *N. lactamicus*, do not grow on the Thayer-Martin medium.

(2) These miscellaneous species, except for *Gemella hemolysans*, are strict aerobes and will not grow anaerobically. *Gemellae* grow equally well aerobically or anaerobically. Increased CO_2 stimulates their growth.

(3) Growth is usually enhanced with the addition of blood or serum to the medium used. Dextrose starch agar without enrichment also gives excellent growth.

(4) Optimum pH for growth is 7.4 to 7.6, and the organisms grow well within a temperature range of 22°C to 35°C.

d. **Isolation:**

(1) Initial cultures should be made on blood agar, on chocolate agar without antibiotic inhibitors, and in thioglycollate broth. If cultures cannot be plated immediately, specimens should be placed in Stuart's transport medium.

(2) Generally, since these organisms are found in areas of heavy contamination, a Gram-stained smear is of no value in making a presumptive identification.

e. **Identification Procedures.** The following tests are recommended for identification of pure culture isolates. Other characteristics and helpful tests are listed in table 6–1.

(1) *Gram Stain.* Look for typical Gram-negative diplococci.

(2) *Oxidase Test.* As a general rule all nonpathogenic species, except for *G. hemolysans*, are oxidase positive. (Tetramethyl salt may give weak positive reactions.) *Gemella*, however, is catalase negative, with varible hemolysis (slow and weak reaction) on rabbit or sheep blood, and attacks sugars fermentatively rather than oxidatively. The oxidase test can further be used for the differentiation of other Gram-negative organisms which may be encountered while culturing for *Neisseria* spp. (see figure 6-3).

(2) *Growth Temperature Test.* The non-

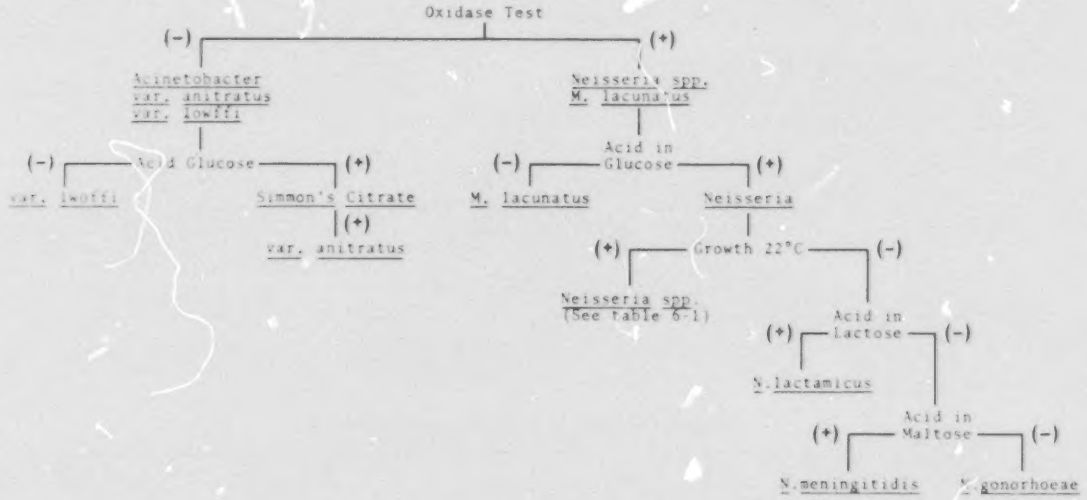


Figure 6–3. Differentiation of Nonreactive Gram-Negative Bacteria (*Acinetobacter*, *M. lacunatus*, and *Neisseria spp*).

pathogenic isolates in most cases grow on nutrient agar at 22° C as well as at 35° C. The pathogenic species generally will not grow at the lower temperature on nutrient agar.

(4) *Carbohydrate Tests.* These tests are performed in cystine-trypticase agar with indicator (BBL or Difco). The agar is supplemented with selected carbohydrates to a final concentration of 1 percent carbohydrate. Inoculum should be heavy and placed on the agar surface. Do not inoculate by stabbing the medium. Incubate at 35° C for 16 to 20 hours and read results immediately after this time period. See table 6–1 for test results. You will notice that *N. lactamicus* is very similar to *N. meningitidis* in sugars utilized, except for lactose. This organism is confusing because it too grows on Thayer-Martin medium. Differentiation can be made on the basis of lactose utilization as *N. lactamicus* is lactose-positive, and by the ortho-nitrophenyl-beta-galactopyranoside (ONPG) test for β-galactosidase. In this test, *N. lactamicus* is positive while *N. meningitidis* is negative.

6–5. The *Veillonella*: Anaerobic Gram-Negative Diplococci:

a. **Taxonomy:**

Part 11.
Family I—Veillonellaceae
Genus—*Veillonella*
Species—*alcalescens; parvula*

b. **Morphology:**

(1) Cells are slightly smaller than the aerobic species and measure 0.3 to 0.4 μ in diameter.

(2) Cells are found arranged in masses, pairs, and short chains.

c. **Pathogenicity:**

(1) The *Veillonella* are normal inhabitants of the mouth, respiratory tract, intestinal tract, and urogenital system of man.

(2) These organisms have occasionally been isolated in disease processes; however, they are considered opportunists rather than true pathogens. Isolates can sometimes be made from blood specimens taken from patients who have had a recent tooth extraction.

(3) Several species are listed in standard reference works, but none are considered to be of great clinical significance.

d. **Growth Requirements:**

(1) The *Veillonella* can be cultured on standard enriched agars, blood agar, chocolate agar, and in rich broth media.

(2) These organisms are strict anaerobes.

e. **Isolation Procedures.** The following procedures are recommended for the identification of pure culture isolates from anaerobic cultures.

(1) *Gram Stain.* Look for typical *Neisseriaceae* morphology.

(2) *Oxidase Test.* The *Veillonella* are oxidase-negative.

(3) *Carbohydrate Tests.* The *Veillonella* do not utilize sugars. They do utilize sodium lactate and pyruvate, producing gas.

(4) *Shake Tubes.* In agar shake tubes the *Veillonella* grow an inch or so beneath the surface and frequently are bluish-green in transmitted light.

6–6. Acinetobacter. This genus of organisms is composed of small, extremely pleomorphic, gram-negative bacteria including *Herellea*, *Mima* and *Bacterium anitratum* under the older taxonomy (Bergey's 1957 ed). In the revised edition of Bergey's (1974), Acinetobacter is inclusive for these organisms, which are recognized as two subspecies of the genus: *A. calcoaceticus*, var. *anitratus* (formerly *Herellea vaginicola* and *B. anitratum*), and *A. calcoaceticus*, var. *lwoffi* (formerly oxidase-negative *Mima polymorpha*). On some occasions, isolates are seen as typical diplococci, and on others as short and long rod forms. When present in the cocoid form, these organisms are easy to confuse with gonococci since the members of the group are saprophytic inhabitants of the female genital tract. Increasingly, isolates which "mimic" the meningococci are obtained from spinal fluid and blood.

a. **Taxonomy:**

Part 10.
Family I—Neisseriaceae
Genus—IV *Acinetobacter*
Species—var. *anitratus*: (*B. anitratus*) var. *lwoffi*: (*Mima lwoffi*)

b. **Morphology.** *Acinetobacter* are extremely pleomorphic. They are seen stained as Gram-negative diplococci in some instances, such as from freshly collected specimens and growth on solid agar media. In broth media and in some liquid specimens, rod forms predominate. Both forms may be seen on the same smear.

c. **Growth Characteristics:**

(1) Good growth is obtained on most enriched media, particularly chocolate agar and blood agar. Colonies on these media are white, smooth, moist, and sticky.

(2) On some occasions isolates are seen as typical diplococci and on others as short and long rod forms. When present in the coccoid form, these organisms are easy to confuse with

gonococci since the members of the genus are saprophytic inhabitants of the female genital tract.

(3) Some of the organisms grow on EMB, MacConkey, and desoxycholate agars but none grow on Salmonella-Shigella agar. Growth rate is variable for the different species on the above media, and this characteristic aids in differentiating them (Consult figure 6-3).

(4) On EMB, *A. calcoaceticus*, var. *anitratus*, produces good growth in 18 to 24 hours and blue-violet colonies. The variety *lwoffi* produces pinpoint colorless colonies after 72 hours.

(5) Members of the genus are inhibited by Thayer-Martin medium.

(6) These organisms are isolated increasingly from debilitated patients having serious underlying diseases, such as cancer, diabetes, etc.

(7) Differentiation of these organisms from the *Neisseria* is important because *Neisseria* are treatable with penicillin but the *Acinetobacter* spp are resistant to this drug, but have a high degree of sensitivity to bacteriocin, which lyses most strains of the organism.

d. **Isolation:**

(1) Make initial cultures on chocolate or blood agar, on EMB, and in thioglycollate or casein digest broth.

(2) Subculture growth to biochemical test media for differentiation.

e. **Identification Procedures:**

(1) Variety *anitratus* forms acid from glucose and variety *lwoffi* does not.

(2) Variety *lwoffi* produces an alkaline reaction on the slant of TSI agar after 24 hours and no hydrogen sulfide is produced. Nitrates are not reduced.

(3) Members of the genus grow well on Simmon's citrate within 24 hours, producing an alkaline reaction. A few strains of each variety yield delayed alkaline reactions and there is an occasional growth with no change in the media.

(4) Acinetobacters may exhibit a variable reaction on urea in Christensen's medium.

(5) Catalase is strongly positive for most members of the genus.

(6) Other carbohydrate tests for differentiation between the variety species *anitratum* and *lwoffi* have been satisfactorily performed in cystine-trypticase medium and O-F medium, utilizing a ratio of 5:1 of carbohydrate to peptone. See table 6-2 for a summary of biochemical characteristics of *Acinetobacter calcoaceticus*, var. *anitratus* (*Herella*) and var *lwoffi* (*Mima*).

Table 6-2. Biochemical Reactions of *Acinetobacter*.

Test or substrate	var. anitratus (Herella)	var. lwoffi (Mima)
	Sign	Sign
Oxidase	-	-
Catalase	+	+
Hydrogen sulfide: TSI agar	-	-
Pb ac paper	-	-
Oxidation fermentation	O	I
Indole	-	-
Methyl red/ Voges-Proskauer	-	-
Motility	-	-
Glucose	+	+
Xylose	+	+
Mannitol	-	-
Lactose	(+)	-
Sucrose		-
Maltose	(+)	-

\+ = 90% or more positive in 1 or 2 days.
\- = no reaction
(+) = 90% or more after 3 days.
I = Inactive
O = Oxidative

Chapter 7

GRAM-POSITIVE BACILLI

SECTION A—CORYNEBACTERIUM DIPTHERIA

7-1. Classification and Morphology:

a. **Taxonomy:**

Part 17.

Family—Corynebacteriaceae

Genus—*Corynebacterium*

Species—*diphtheriae*
ulcerans
haemolyticum
equi
aquaticum
acnes (*Propionibacterium*)
xerosis
vaginale (Present taxonomy, 1974)

Genus—*Listeria*

Species—*monocytogenes*

Genus—*Erysipelothrix*

Species—*rhusiopathiae* (*insidiosa*)

Genus—*Microbacterium*
Cellulomonas
Microbacterium
Arthrobacter

b. **Morphology:**

(1) *Corynebacteria.* Corynebacteria are morphologically slender, nonsporulating rods appearing "club shaped" and irregular. They are gram-positive and measure 0.5 to 1 μm in diameter and several μm in length. In stained smears the bacteria are arranged in palisades, or what is called a "Chinese letter" pattern, because they sometimes resemble Chinese character script. Irregularly distributed within the rod-shaped organisms are deeply stained granules. The intense color is obtained by using aniline dyes. The metachromatic granules produced are called Babes-Ernst bodies and give the rods a beaded appearance when viewed macroscopically.

(2) *Listeria monocytogenes. Listeria monocytogenes* is morphologically a nonspore-forming rod with rounded ends. It is gram-positive and measures 0.5 to 0.6 μm in length. In gram-stained smears the bacilli form "palisades." They are morphologically similar to "diphtheroids" (harmless saprophytes), which are usually short, thick, uniformly stained rods in palisade arrangement, exhibiting little or no pleomorphism. *Listeria* differs also by being motile by means of peritrichous flagella. It is different from *C. diphtheria* in that metachromatic granules are generally not observed in stained preparations.

(3) *Erysipelothrix rhusiopathiae* (*insidiosa*). *E. rhusiopathiae* is a slender gram-positive rod measuring about 0.2 to 0.4 μm by 0.4 to 2.5 μm. The rods are straight or curved, with rounded ends. Basically, *Erysipelothrix* is morphologically similar to *Listeria.*

7-2. Pathogenicity. Disease-causing Corynebacteria are found on the surfaces of mucous membranes in man and lower animals, on plants, and in soil. Those species which are of interest to man include *C. ulcerans, C. equi, C. haemolyticum, C. vaginale,* and *C. aquaticum.*

a. **Diphtheria Bacilli.** *Corynebacterium diphtheriae,* varieties *gravis, intermedium,* and *mitis,* are causative organisms in the disease diphtheria. This disease is contagious. The bacilli are spread by moist, airborne particles from asymptomatic carriers or infected individuals. The organism invades the mucous membranes of the upper respiratory tract, producing a powerful exotoxin. This toxin is absorbed by the mucous membrance, causing an inflammatory response that destroys the epithelium. *C. diphtheriae* causes the formation of a pseudomembrane and a nerve toxin in the course of the disease diphtheria.

(1) *Pseudomembrane.* This is a grayish substance which often covers the tonsils, pharynx, or larynx during the course of the disease. This membrane is formed by an accumulation of leukocytes, erythrocytes, and fibrin deposits.

(2) *Nerve Toxins.* Nerve poisons or neurotoxins produced by *C. diphtheriae* are capable of disabling the soft palate, eyes, heart muscles, etc. The effect of the disease on heart muscle is perhaps the most dangerous aspect of the disease. The total effect depends on the time the disorder goes untreated. The bacteria remain in the lungs and disseminate their toxins by way of the bloodstream. This toxin is a powerful exotoxin.

(3) *Toxin Variation.* The three varieties of diphtheria produce essentially the same toxin; therefore, an antitoxin to any one of the varieties gives immunity to all three.

(4) *Anaerobic Diphtheroids, Propionibacterium* (*Corynebacterium*) *Acnes: C. liquefaciens.* Anaerobic diphtheroids that regularly survive on normal skin are thought to be par-

ticipants in the pathogenesis of acne. The bacteria produce a lipase, which splits off free fatty acids from skin lipids. These fatty acids can cause tissue inflammation and contribute to the acne disorder.

b. **Other Corynebacterium Species.** There are other species of Corynebacteria which have a low percent of occurrence as compared to the diphtheria species of the genus. The number of Corynebacteria species recognized as pathogenic to man and animals has increased to include the following: *C. ulcerans, C. haemolyticum, C. equi, C. bovis, C. aquaticum,* and *C. xerosis.* These species have been known for some time but not implicated in diseases of man. Their clinical involvement continues to be low, but should be mentioned. *C. ulcerans* and *C. haemolyticum,* for example, are more prevalent than in previous years. *C. ulcerans* has been isolated from infections of the tonsils, and *C. haemolyticum* from throat, blood, and skin infections. *C. equi* and *C. bovis,* which are thought to be predominantly pathogens of animals, have been isolated from blood, urine, cerebrospinal fluids, and abscesses in man. *C. aquaticum* is found in its natural habitat, water, but is also recovered from human sources, such as blood, spinal fluid, and sputum. *C. vaginale* (*H. vaginalis*) is associated with nonspecific urethritis and vaginitis, but may be part of the normal vaginal flora. *C. renale* has been isolated from purulent infections of the urinary tract in cattle, sheep, horses, and dogs, but is rarely encountered in human infections. *C. xerosis* has been reportedly isolated from the skin surface and the eyes.

c. **Listeria.** Very little is known about *Listeria.* It is only on rare occasions that the organism has been isolated in conjunction with disease in man. The disease listeriosis or "glandular fever," caused by *L. monocytogenes,* is an acute benign lymphoblastosis commonly found in children and young adults. On occasion, *L. monocytogenes* has been associated with meningitis, septicemia, and abortions.

d. **Erysipelothrix.** *Erysipelothrix rhusiopathiae* (*E. insidiosa*) is the causative agent of the disease erysipeloidosis and has a predilection for the skin, joints, and endocardium. The disease is primarily an occupational one affecting individuals who handle fish, shellfish, poultry, and certain meat products, particularly pork.

7–3. Growth Requirements:

a. **Corynebacteria** grow on most routine bacteria media. On Loeffler's serum they grow much more rapidly than do other respiratory pathogens, staining to conform to typical morphological description.

(1) On carbohydrates, fermentation reactions are variable, but most species oxidize glucose to form CO_2 and H_2O but no gas. Carbohydrate reactions are shown in table 7–1.

(2) Corynebacteria organisms are aerobic and develop well at 35°C, requiring about 24 hours for growth on Loeffler's (coagulated serum) or Pai's (coagulated egg) medium. Not all species of Corynebacteria are supported on potassium tellurite, but in about 48 hours, *C. diphtheria* tends to turn a characteristic gray or black color, which helps in differentiating

Table 7–1. Carbohydrate Reactions of *Corynebacterium.*

	Glucose	Sucrose	Maltose	Starch	Nitrate	Urease	Catalase	Toxigenic	Hemolysis
C. diphtheriae	+	-	+	±	±	-	+		
C. ulcerans	+	-	+	+	-	+	+		
C. haemolyticum	+	±	+	-	-	-	-		
C. hoffmanii	-	-	-	-	±	+	+		
C. xerosis	+	+	+	-	+	-	+		

the species.

b. **Listeria.** This organism is a facultative anaerobe and grows well aerobically in a neutral or slightly alkaline medium. On blood agar, Listeria grows very well, producing a narrow zone of hemolysis. Specimens for culture may require refrigeration at 4°C (as tissue suspensions, fluids, etc) up to 90 days prior to incubation to improve chances of recovery. Listeria will also grow on sheep liver extract agar plates or slants. The optimum temperature for cultivation of Listeria is 35°C.

c. **Erysipelothrix.** *E. rhusiopathiae (insidiosa)* grows poorly on simple media; however the addition of serum or glucose enhances growth.

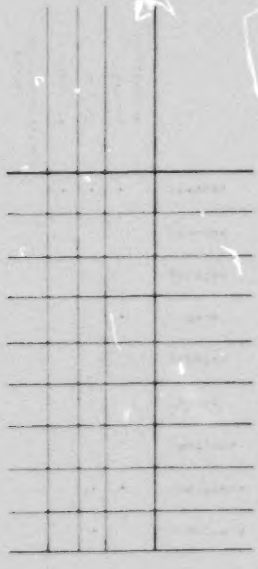
7-4. Cultural Characteristics:

a. **Corynebacteria.** Saprophytic organisms *Corynebacterium diphtheroides* (diphtheroids) (*Nomen dubium*), which normally inhabit the mucous membranes of the respiratory tract, generally grow more abundantly on blood, Loeffler's and tellurite agars than does the diphtheria bacillus. Colony size is not necessarily a criterion for differentiating between pathogenic and saprophytic forms. Table 7-2 lists the colony characteristics for diphtheroids and the varieties of *Corynebacterium diphtheriae*.

b. **Listeria.** *Listeria* produces beta hemolysis; this, however, is not a constant characteristic. On blood agar (sheep, human, or rabbit) the colonies are small and gray to white, measuring 0.8 to 2.0 µm in diameter. Hemolysis may be present after 48 hours incubation on human or rabbit blood, but on sheep blood, it may occur within a 24-hour period. On potassium tellurite medium, the colonies are black, circular, and smooth, measuring about 0.25 to 0.5 µm in diameter. On routine media, both rough (R) and smooth (S) colony types may be noted.

c. **Erysipelothrix.** *Erysipelothrix* exhibits rough colonies at 35°C, while smooth colonies are produced at 33°C. After a 24-hour incubation period, the small, rough colonies have a matte surface with edges similar to anthrax colonies. The smooth colonies are round, water clear, and glistening. Many of these colonies produce an alpha hemolysis on blood agar after prolonged incubation. The genus *Erysipelothrix* is facultatively anaerobic, although it tends to grow microaerophilically. The organism also is catalase negative.

Table 7-2. Criteria for Differentiation of *Corynebacterium* spp (*diphtheriae* var).



7-5. Identification:

a. **Corynebacterium:**

(1) *Obtaining Specimens.* Obtain swabs from the nose, throat, or other suspected lesions before antibiotics are given the patient. This precaution is necessary because the presence of an antibiotic in a specimen may inhibit growth of the organism.

(2) *Handling Specimens:*

(a) Prepare smears and stain them with alkaline methylene blue (figure 7-1) or Gram's stain to determine morphology (see paragraph 7-1b).

(b) Prepare cultures for further studies, using appropriate media (see paragraph 7-4).

(c) Final identification can be accomplished only by careful studies of cultures and demonstration of exotoxin production.

(3) *In Vitro Test:*

(a) Immerse sterile strips of filter paper (1×7 cm) in diphtheria antitoxin (500 units per ml).

(b) Prepare agar containing KL virulence enrichment. Pour the KL agar into sterile petri dishes. Immediately place a filter paper strip as prepared in (a) above on the melted agar. Prod the filter strip with sterile forceps so that the strip settles beneath the agar surface.

(c) After the media has solidified, streak a loopful or organisms in a single line over the agar surface at right angles to the filter strip.

(d) Streak a known toxigenic *C. diphtheria* organism on the same plate to serve as a control.

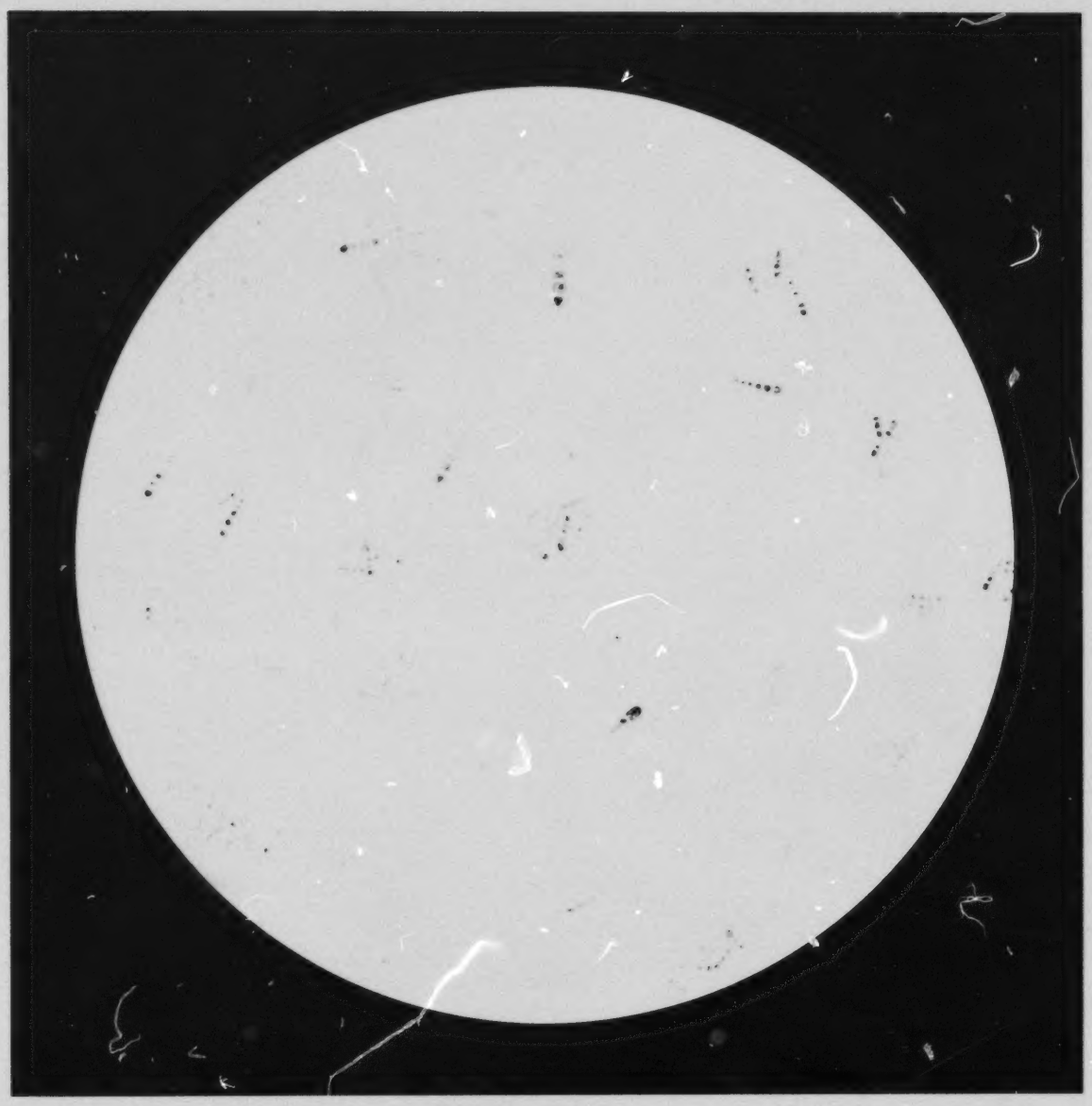


Figure 7–1. Loeffler's Alkaline Methylene Blue Strain of *Corynebacterium diphtheriae* From Loeffler's Serum Medium.

(e) Incubate at 35°C for 72 hours. Examine the plates at 24-hour intervals.

(4) *Interpretation.* If the *Corynebacterium* sp is toxigenic, white lines of precipitate will be observed in the agar. The lines will appear about 1 cm from the filter strip and will extend from the line of growth at a 45° angle. The lines of precipitate, evidence of a specific toxin-antitioxin reaction, will be easily observed over a dark background under oblique lighting. Nontoxigenic strains of Corynebacteria will not exhibit lines of precipitation.

b. **Listeria:**

(1) Incubate media (see paragraph 7–4b) for 24 hours at 35°C.

(2) Inspect under a magnifying lens for very small colonies.

(3) Colonies may appear blue-green in color on phenyl-ethanol agar (PEA).

(4) To demonstrate motility of Listeria, use a dextrose, semisolid, agar-stab culture.

(a) Inoculate two tubes: one at 35°C

and the other at 25°C. Motility in the agar is more pronounced at 25°C.

(b) In motile colonies, a ring forms just beneath the surface of the agar; a cloudlike growth then extends downward from the ring.

(5) In examining blood agar plates, take care not to confuse colonies giving beta hemolysis with beta hemolytic streptococci (*pyogenes*). A stained slide will show the differences in morphology.

(6) *Listeria* grows on tellurite agar and must not be mistakenly discarded as a diphtheroid. The colonies are small, black, and glistening, surrounded by a characteristic zone of green coloration. Differentiation can be made through use of Esculin (hydrolyzed by *Listeria* motility, and gram stains (to distinguish *Listeria* from *Corynebacteria*).

c. **Erysipelothrix.** Provisional diagnosis of erysipeloidosis is based primarily on clinical findings, but final diagnosis rests on the isolation of *E. rhusiopathiae* (*insidiosa*) from the lesion. Biopsies of the lesion should be taken for culture, because positive cultures are rarely obtained by swabbing the infected area.

(1) Initial cultivation should be started in a glucose broth under carbon dioxide tension with subsequent subculture to blood agar plates. In a gelatin stab the growth is filiform at first; then lateral radiating growth resembling a "test-tube brush" appears.

(2) On gelatin slants, the smooth (S) forms remain along the line of inoculation, whereas the rough (R) forms spread out over the surface.

(3) The principal distinguishing characteristics of *Erysipelothrix* are that it is catalase negative and that it produces H_2S in KIA or TSI tube slants. Table 7–3 shows differentiation within the family *Corynebacteriaceae*.

7–6. Key for Identification. Table 7-4 shows the morphological comparison of the *Corynebacterium* species. This table also shows colony differentiation within the genus *Corynebacterium*.

SECTION B—GRAM-POSITIVE AEROBIC SPORE-FORMING BACILLI

7–7. Classification and Morphology:

a. **Taxonomy:**

Part 15.
Family I—Bacillaceae
Genus I—*Bacillus*
Species—*anthracis*
cereus

b. **Morphology:**

(1) Anthrax bacilli are large, pathogenic bacteria, 1 to 2.5 μm in width and 3 to 10 μm in length. In smears, the anthrax bacilli appear as long, straight-sided rods with curved, swollen, or concaved ends, resembling *Bacillus cereus*. There is a bamboo-like appearance to the stained organism.

(2) Anthrax is a gram-positive organism which forms spores when conditions are unfavorable for characteristic growth. Capsules surround the organisms as they grow in the blood or tissue of infected animals.

(3) When examined in smears from the blood or tissues of an infected animal, the organisms usually are found singly or in pairs.

7–8. Pathogenicity:

a. Anthrax is not primarily a disease of man, but of animals, such as cattle, sheep, and rarely horses, hogs, and goats.

b. The infection usually acquires entry through injuries in the skin or membrances of the animal. The portal of entry in animals is usually through the mouth and gastrointestinal tract. Spores find easy access when ingested with spring vegetation. Man is infected by way of inhalation of spores or through breaks in the skin, usually cuts, scratches, or small abrasions.

c. Anthrax is perhaps not too prevalent today but has greater importance in areas where man and animal come in frequent contact.

d. Spores enter into the tissue and germinate at the site of entry, resulting in the formation of a gelatinous edema and congestion. Later

Table 7–3. Differentiation of *Corynebacterium*, *Listeria*, and *Erysipelothrix*.

Genus	Catalase	Motility	Metachromatic Granules	Oxygen Requirements
Corynebacterium	+	-	Usually present	Aerobic Facultative anaerobe
Listeria	+	+	Usually absent	Aerobic Facultative anaerobe
Erysipelothrix	-	-	Usually absent	Microaerophilic

Table 7–4. Colony Characteristics of *Corynebacteria*.

Species	*Blood agar (18–24 hours)*	*Loeffler's serum medium (18–24 hours)*	*Potassium tellurite agar (48 hours)*
C. diphtheriae, var. *gravis*	Small, gray, dull opaque colonies which are non-hemolytic.	Circular, convex, cream-colored colonies with raised centers.	Flat, irregular, slate-gray colonies with a dull surface (2–3 mm in diameter).
C. diphtheriae, var. *intermedius*.	Small, gray, dull opaque colonies which are non-hemolytic.	Similar to var. *gravis*	Circular, convex colonies with brownish-gray color against a white background (0.2–0.3 mm in diameter).
C. diphtheriae, var. *mitis*	Small, gray, dull opaque colonies which are usually hemolytic.	Similar to var. *gravis*	Black, convex colonies with a glistening surface (1.0–1.5 mm in diameter).
Diphtheroid bacilli	*Colonies considerably variable and nonhemolytic.	Similar to var. *gravis*	Flat colonies, light gray or dark in center with white or gray translucent periphery.

*Certain diphtheroids form colonies that are dull and flat with a slightly rough surface. Others form staphylococcus-like colonies that are large, grayish white and smooth surfaced. Yellowish pigmented forms resembling *Neisseria* spp. are occasionally encountered.

the disease spreads to the lymphatics, the blood stream, and deeper tissues.

e. Infections may be named according to the site: cutaneous, pulmonary, or intestinal anthrax.

7–9. Growth Requirements:

a. *B. anthracis* requires no special additives to the media. It grows well on routine laboratory media.

b. It is strictly aerobic and grows well at 35°C (pH 7.0 to 7.4).

c. Under CO_2 conditions some strains of anthrax bacilli will become encapsulated. Some colonies growing under these conditions are not capable of producing disease.

7–10. Cultural Characteristics:

a. *B. anthracis* produces nonhemolytic colonies having a "cut-glass" appearance in transmitted light with dull, irregular edges.

b. *B. cereus* and B. subtilis, the nonpathogens, produce greenish-yellow, clear hemolysis on blood agar. The medium turns very dark with growth of "rough" wrinkled colonies.

c. When gelatin is stabbed with anthrax and incubated at 25°C, liquefication occurs, and growth resembles an "inverted fir tree."

7–11. Identification:

a. In gram stains of smears of exudate and blood, *B. anthracis* appears as large, gram-positive, chaining cells.

b. Proper handling in collecting specimens for investigation is important to the technician and the patient. Avoiding contamination with common *Bacillus sp* prevents confusion in the identification of the pathogen.

c. The Wirtz-Conklin procedure is a stain used to demonstrate the spores within the cells (figure 7–2). The technique employs heat or tergitol (surface-active agent) to stain the bacillary cells and spores. Autoclaving suspected samples is a must to prevent contamination with this very dangerous organism.

7–12. Key for Identification. Table 7-5 is provided as an aid in the differentiation of *B. anthracis* from Pseudoanthrax (saprophytic *Bacillus spp*).

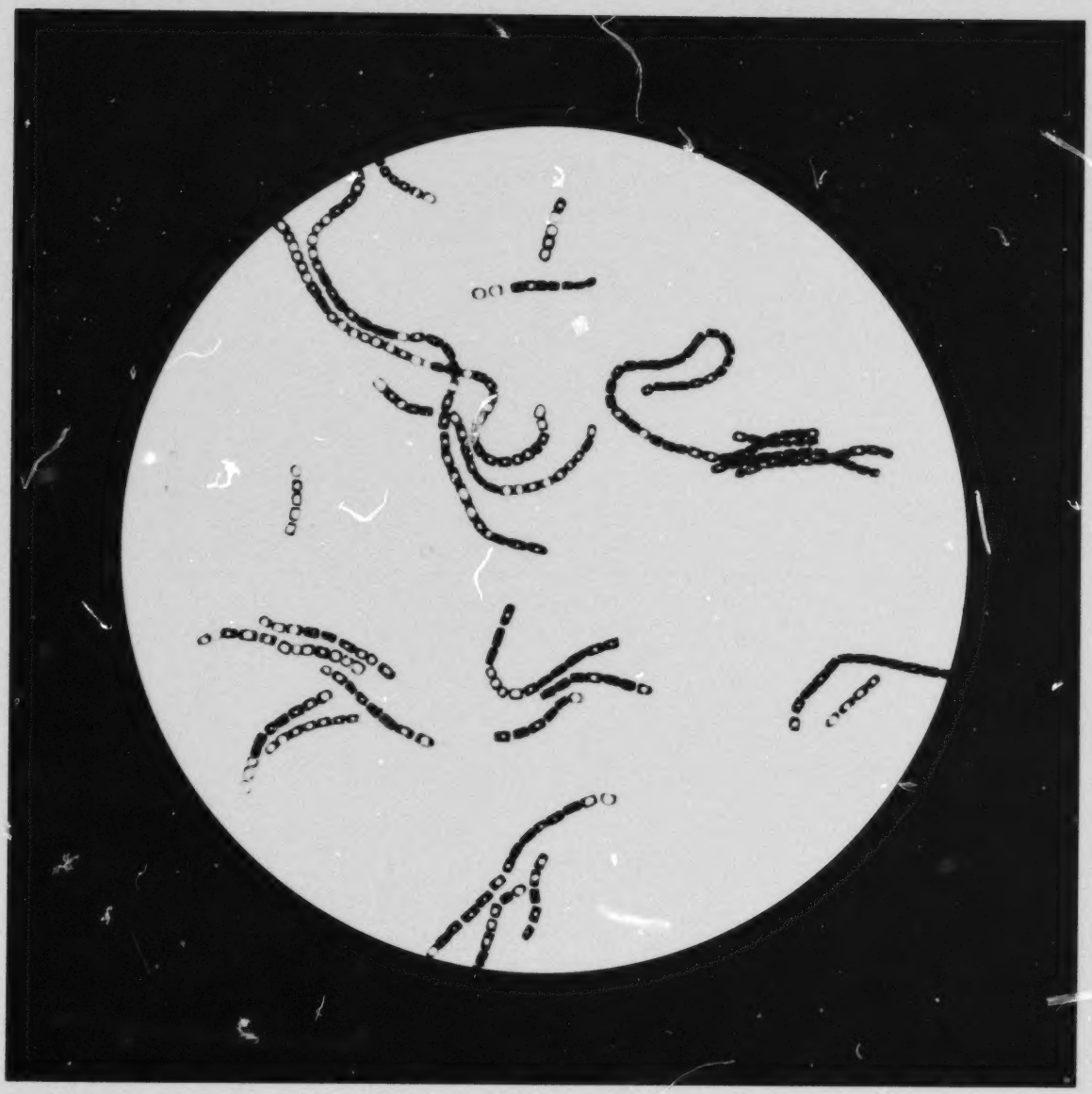


Figure 7–2. Writz-Conklin Spore-Stained Smear of *Bacillus anthracis* From Infusion Agar Medium.

SECTION C—GRAM-POSITIVE ANAEROBIC SPORE-FORMING BACILLI

7–13. The Genus *Clostridium*:

a. **Taxonomy:**

Part 15.

Family I—Bacillaceae

Genus III—*Clostridium*

Species—*perfringens*
tetani
botulinum
bifermentans
butyricum
innocuum
septicum
sordellii
sporogenes
novyi
histolyticum
haemolyticum

b. **The genus Clostridium** contains over 150 species, many of which are of interest to man.

Table 7–5. Differentiation of *Bacillus anthracis* From Saprophytic (Pseudoanthrax) Bacillus spp.

Organism	*Colonies on blood agar*	*Hemolysis*	*Motility*	*Capsule*	*Mouse or Guinea Pig virulence*
Virulent *B. anthracis*	Rough, dull, and irregular ("frosted glass")	−	−	+	+
Avirulent *B. anthracis*	Rough, dull, and irregular ("frosted glass") or smooth or mucoid.	−	−	−	−
Saprophytic (pseudoanthrax) *Bacillus* spp.	Rough, dull, flat, irregular and often spreading. Occasionally smooth, shiny and compact.	+ (usually)	+ (usually)	−	−

Several of the species decompose protein or ferment carbohydrates. Also, there are several pathogenic species which produce toxins. The majority of the species are saprophytic and do not produce a toxin. Clostridia are always present in soil and in the intestinal tracts of man and animal. In the soil they decompose organic matter and in the intestinal tract, perform a similar action. The pathogenic Clostridia include toxin-producing, noninvasive saprophytes (for example, *C. botulinum*), slightly invasive organisms which produce a potent toxin (for example, *C. tetani*), and also organisms which are invasive and cause an enterotoxemia and gangrene in involved tissues (for example, *C. perfringens*).

c. **Morphology.** All species consist of relatively large gram-positive rods. They also form spores, which are usually wider in appearance than the cell that contains it. The location of the spore within the cells may vary from species to species and is used in the identification and classification of the organisms. Consider the morphology of these common species of the genus: *C. perfringens, C. tetani,* and *C. botulinum.*

(1) *C. perfringens* is a short, plump, sporebearing, gram-positive bacillus, occurring singly or in pairs. It does not form chains as a rule. It is nonmotile and has a capsule.

(2) *Clostridium tetani* is a slender rod 2 to 5 μm in length and 0.3 to 0.8 μm in width. Peritrichal flagella are present in the younger organisms. Spores located at the ends give the appearance of a "drumstick."

(3) *Clostridium botulinum* is a gram-positive rod with rounded ends, 3 to 8 μm in thickness. The bacillus is slightly motile and possesses from four to eight flagella.

7–14. Pathogenicity:

a. **Clostridium perfringens** is associated with gas gangrene. Other species which have been isolated from cases of gangrene are *C. sporegenes, C. bifermentans* (*C. sordellii*), and *C. novyi. C. perfringens* is a normal inhabitant of the intestinal tract of man and animals, and becomes pathogenic only in cases of intestinal perforation. Any wound contaminated with soil also may be infected with these spore-bearing anaerobes. *C. perfringens* can cause food poisoning, due to its heat-resistance through spore formation and its ability to produce a toxin in the foodstuff. Recovery from food poisoning may occur in 24 hours or less without hospitalization, except in cases of elderly patients who may not recover.

b. **C. tetani** is the cause of tetanus, sometimes called lockjaw. *C. tetani* is a very virulent organism producing a fibrinolysin, a hemolysin, and other toxins.

(1) The fibrinolysin is capable of destroying fibrin formed by the body's coagulation mechanism.

(2) The hemolysin causes hemolysis of erythrocytes, releasing hemin into the bloodstream.

(3) Toxins produced by *C. tetani* are specific for the nervous system and are known as "neurotoxins." Neurotoxins are extremely potent, having an average specific activity of 150 $\times 10^6$ minimum lethal dose per mg of N.

(a) Human beings are extremely susceptible to the tetanus neurotoxin. Cases have

been known in which men have died from only a scratch by a needle used for injecting a horse with toxin (horse used to produce antitoxin).

(b) The toxin is selective, acting upon the nerve cells of the cerebrospinal axis as well as the medulla oblongata.

(4) Antitoxin is produced by first treating the toxin with formalin, converting it to a toxoid, and then injecting human volunteers. The toxoid is nontoxic but still antigenic. The human antitoxin is more potent than that produced by animals and is not destroyed by immune mechanisms of the patient.

c. **C. botulinum** is the cause of "food poisoning." The significant metabolite here is a very potent exotoxin, of which there are six known types designated as A through F.

(1) The spores of types A and B can resist heat better than any of the other clostridial anaerobes. The toxins produced act on the myoneural functions and produce death by respiratory paralysis while type E produces acute gastrointestinal symptoms which may lead to incorrect diagnosis of obstruction of the bowels.

(2) *C. botulinum* and *C. parabotulinum* (*C. botulinum* type A) found in fresh foods, cooked or raw, cause food poisoning. Cases following the ingestion of home-canned vegetables and fruits have a mortality rate of about 70 percent.

7–15. Growth Requirements. The Clostridia are obligate anaerobes. Growth may be obtained over a wide range of temperature; however, 35°C is optimum for pathogenic species. Although variations in nutritive requirements exist throughout the Clostridia, the organisms are successfully isolated from specimens, using blood agar and thioglycollate broth (containing 0.6 percent glucose). Anaerobic conditions may be provided by incubating the inoculated blood agar plates in a Brewer anaerobic jar. The Clostridia grow well in the depths of thioglycollate broth, in a normal atmosphere, without the necessity of providing an airtight seal for the mouth of the culture tube. Clostridial organisms grow well on most routine laboratory media upon incubation in an anaerobic (low oxidation-reduction potential) environment. Conditions favorable to growth of *C. perfringens*, *C. tetani*, and *C. botulinum* are discussed below in some detail.

a. **Clostridium perfringens** grows under strictly anaerobic conditions in media containing tissue, such as meat slightly cooked. Air should be expelled from tubes by boiling. Good growth also can be obtained using milk placed in tubes (sterile) in an anaerobic jar.

(1) *C. perfringens* does not form spores readily.

(2) The need to grow under anaerobic conditions is not as great as for *C. tetani*, since *C. tetani* will grow when air pressure is between 0 to 4.5 mm Hg and the former will not.

b. **C. tetani** is an obligate anaerobe which loses its ability to produce a toxin when grown microaerophilically.

(1) Excellent growth is obtained with the use of thioglycollic acid on fluid medium. In media retaining residual amounts of oxygen after preparation, the deleterious effects of oxygen can be neutralized, and growth can be obtained by the addition of proper chemicals to reduce the oxygen tension.

(2) Conversely, this fact of increased oxygen tension, inhibiting toxin production, is utilized in the treatment of tetanus infections, and gas gangrene by the use of hyperbaric oxygen therapy. Patients are placed in a pressure chamber where oxygen tension can be increased under controlled conditions.

c. **Clostridium botulinum** is a strict anaerobe and is easily cultivated under anaerobic conditions on the usual meat-infusion media. Several types of *C. botulinum* have been isolated. They have been designated as type B *C. botulinum*, type C (*Bacillus botulinus*), type D (*C. parabotulinum*), and type E (*C. botulinum*). Corresponding toxins, designated A through E, are produced by these organisms when grown at optimum temperatures, which in most cases range between 25°C and 37°C.

(1) The organism is extremely susceptible to the reaction of the medium, growing only at neutral or moderate alkalinity.

(2) Treatment with penicillin and lysozyme converts the growing cultures into protoplasts (a mass of protoplasm), which produce no toxin and therefore are harmless.

(3) Outbreaks of botulism are rare in the United States because of the rigid regulation of commercial canning and food preservation. The only cases since 1910 have been those associated with home preparation of foodstuffs.

7–16. Cultural Characteristics. On blood agar after 48 hours of anaerobic incubation at 35°C, typical colonies of the various *Clostridium spp* appear, as shown in table 7–6. Many of the pathogenic species, such as *C. septicum*, *C. sporogenes*, *C. tetani*, and *C. perfringens*, exhibit the ability to hemolyze blood in culture. A characteristic double-zone or target-appear-

Table 7-6. Distinguishing Characteristics of *Clostridium* Species.

	Casein Digested	Lecithinase	Indole	Lipase	Glucose	Mannose	Maltose	Lactose	Salicin
C. perfringens	-	+	-	-	+	+	+	+	v
C. tetani	d	-	v	-	-		-	-	-
C. botulinum	+	-	-	+	+	-	v	-	v
C. butyricum	-	-	-	-	+	+	+	+	v
C. innocuum	-	-	-	-	+	+	v	-	+
C. septicum	-	-	-	-	+	+	+	+	v
C. sordellii	+	+	+	-	+	v	+	-	-
C. sporogenes	+	-	-	+	+	-	v	-	d
C. novyi (Type A)	-	+		+	+	-	d	-	-
C. histolyticum	+	-	-	-	-	-	-	-	-
C. haemolyticum	-	+	+	-	+	v	-	-	-

v = variable
d = reactions differ
(-) = negative
(+) = positive

ing hemolysis is caused by *C. perfringens* when cultured on blood agar under anaerobic conditions. Although the microscopic and colonial morphology of certain Clostridia may appear quite distinctive, final identification should be made by the utilization of biochemical tests, gas chromatography, and animal toxicity studies.

7-17. Identification. When spores of a possible *Clostridium* spp are observed in a mixed thioglycollate broth culture, their resistance to heat may be used advantageously in obtaining isolation. One-tenth millimeter of the mixed culture is aseptically inoculated to a fresh tube of thioglycollate broth. The freshly inoculated broth is then heated in a water bath at 80°C for 15 to 30 minutes. This will destroy all vegetative growth of bacteria, but will not destroy the spores. The heated medium is then incubated for 24 to 48 hours, resulting in a pure culture of the *Clostridium* species.

a. **Clostridium perfringens.** In cases of gas gangrene, exudate from wounds may be collected with a sterile swab or syringe, for staining or culture.

(1) Check the culture carefully daily.

(2) Check smears for the presence of large gram-positive, sporeforming rods.

b. **Clostridium tetani.** In clinical cases, diagnosis depends on the clinical history and the nature of the injury. Therapy should not be withheld until the presence of organisms is demonstrated. Again, the use of cultures and smears is helpful in identification and demonstration of the causative agent.

c. **Clostridium botulinum.** Toxins from organisms can occasionally be demonstrated in the serum of patients, as well as in leftover foods. *C. botulinum* may be grown from bits of the remaining food. When isolated colonies are obtained on blood agar, the growth should be carefully picked and transferred to thioglycollate broth. Following incubation, this pure culture is used to demonstrate toxin production.

(1) Mice may be injected with toxin intraperitoneally, causing rapid death.

(2) Botulinum toxin can occasionally be demonstrated in food by a hemagglutination technique (agglutination of coated erythrocytes with a specific antiserum).

7–18. Key for Identification. Table 7–7 is provided as a simplification of the morphological and biological differences of the various species of Clostridia.

Table 7-7. Morphological and Biochemical Differentiation of *Clostridium* Species.

Species	*Surface colonies anaerobic blood agar*	*Motility*	*Spores*	*Fermentation (deep agar)*			*Indole Production*	*Nitrate reduction*	*Proteolysis (cooked meat)*
				Dextrose	*Lactose*	*Sucrose*			
Cl. tetani	Small (1 mm) flat, rhizoid or feathery surface; raised centers; single hemolytic zone.	+	Round, terminal, and swollen (drumstick).	−	−	−	−	−	Gas.
Cl. botulinum	Large (5–10 mm); glistening translucent edges with brown centers; single hemolytic zone.	+	Oval, subterminal and swollen.	+	−	−	−	−	Gas, digestion blackening.
Cl. perfringens	Large (2–5 mm), round, smooth, opaque, entire; double hemolytic zones.	−	Oval, central, nonswollen (rarely present).	+	+	+	−	+	Gas.
Cl. novyi	Small, delicate, flattened, transparent; blue gray and irregularly contoured; single hemolytic zone.	+	Oval, eccentric, subterminal and swollen.	+	−	−	−	−	Gas.
Cl. septicum	Small, with branching or rhizoid margins and deep, opaque centers; single hemolytic zone.	+	Oval, subterminal, swollen.	+	+	−	−	+	Gas.
Cl. sporogenes	Small, transparent, with rhizoid or amoeboid margins and raised centers; single hemolytic zone.	+	Oval, eccentric, subterminal and swollen.	+	−	−	−	−	Gas, digestion blackening.
Cl. chauvoei	Small, flat, round or leaflike; single hemolytic zone.	+	Oval, eccentric, subterminal and swollen.	+	+	+	−	+	Gas.
Cl. histolyticum	Minute, round, translucent, with "dew drop" appearance; single hemolytic zone.	+	Oval, subterminal and swollen.	−	−	−	−	−	Digestion blackening.
Cl. bifermentans	Small, transparent, with rhizoid margins; single hemolytic zone.	+	Oval, eccentric, subterminal and swollen	+	−	−	+	−	Gas, digestion blackening.

Chapter 8

GRAM-NEGATIVE BACILLI: ENTERIC

8-1. Introduction. In the intestinal tract of man we find members of family Enterobacteriaceae, making up a large part of the aerobic bacterial flora. The intestinal flora include *Escherichia, Klebsiella, Enterobacter, Proteus, Pseudomonas,* and members of the genus *Providencia.* The pathogenic enterics of the adult intestinal tract include members of the genera *Salmonella, Shigella, Arizona* (*A. hinshawii*), and *Edwardsiella* (*E. tarda*). Care should be practiced in handling of all specimens because all harbor potentially pathogenic organisms. Many saprophytic gram-negative organisms of the intestines cause infection when allowed to grow in other areas of the body (for example, the ear, genitals, lungs, and spinal column). Infant diarrhea is caused by "saprophytic normal flora" *Escherichia coli. Proteus* strains also have been implicated in some outbreaks of infant diarrhea.

8-2. Classification and Morphology:

a. **Classification.** The bacteria considered in this chapter belong for the most part to the family Enterobacteriaceae, but a few belong to the families Vibrionaceae and Pseudomonadaceae. At the beginning of each section the taxonomic position of the genus is shown. Classification is based on certain similarities between organisms, such as morphology, motility, and sugar utilization.

b. **Morphology.** Morphologically, enterics are very similar. They all stain gram-negative, are rod shaped, and are 1.0 to 3.5 μm in length and 0.5 to 0.8 μm in width. Their arrangement as seen microscopically may vary from short to plump rods, arranged singly, in pairs, in clumps and occasionally in short chains. However, microscopic morphology is of little diagnostic value in most cases because of similarity between members of the Family Enterobacteriaceae.

8-3. Examination of Cultures:

a. **Colony Characteristics.** Laboratory technicians should become familiar with the colony characteristics of enteric bacilli on blood agar as well as on commonly used plating media for stool examination. Enteric organisms cultivated on blood agar usually reveal large, smooth shiny, circular, raised colonies which may or may not be hemolytic or pigmented. *Proteus* spp often exhibit swarming.

b. **Pigmentation.** Colorless colonies on differential and selective media indicate the organism is a nonlactose* fermenter (possible pathogen) and must be identified by further studies given in this chapter. Colored colonies indicate the organism is a lactose fermenter. Figures 8-1 and 8-2 are flowcharts summarizing reactions of lactose and nonlactose fermenters. Organisms producing colored colonies on enteric media are usually nonpathogenic for adults, providing these organisms have been isolated from their normal habitat—the intestinal tract. Certain lactose fermenting enteric organisms may be etiological agents in cases of infant diarrhea. Methods for identification are given in this chapter.

c. **Preliminary Studies.** Preliminary studies for enteric identification are usually biochemical in nature. These biochemical studies should be used to provide a tentative identification of the genus. Final identification of enteric gram-negative bacilli is usually dependent upon the following studies: (1) serological evaluation to confirm genus and assign tentative species designation, and (2) biochemical confirmation of genus and species.

8-4. Preliminary Biochemical Tests:

a. Inoculate typical isolated colonies from differential or selective media to the following media:

(1) Citrate.

(2) Motility (SIM medium may be used to combine indole and motility tests).

(3) Tryptone broth (indole test).

(4) Triple sugar iron agar.

(5) Urea (hydrolysis of urea).

(6) Clark and Lub broth (methyl red test).

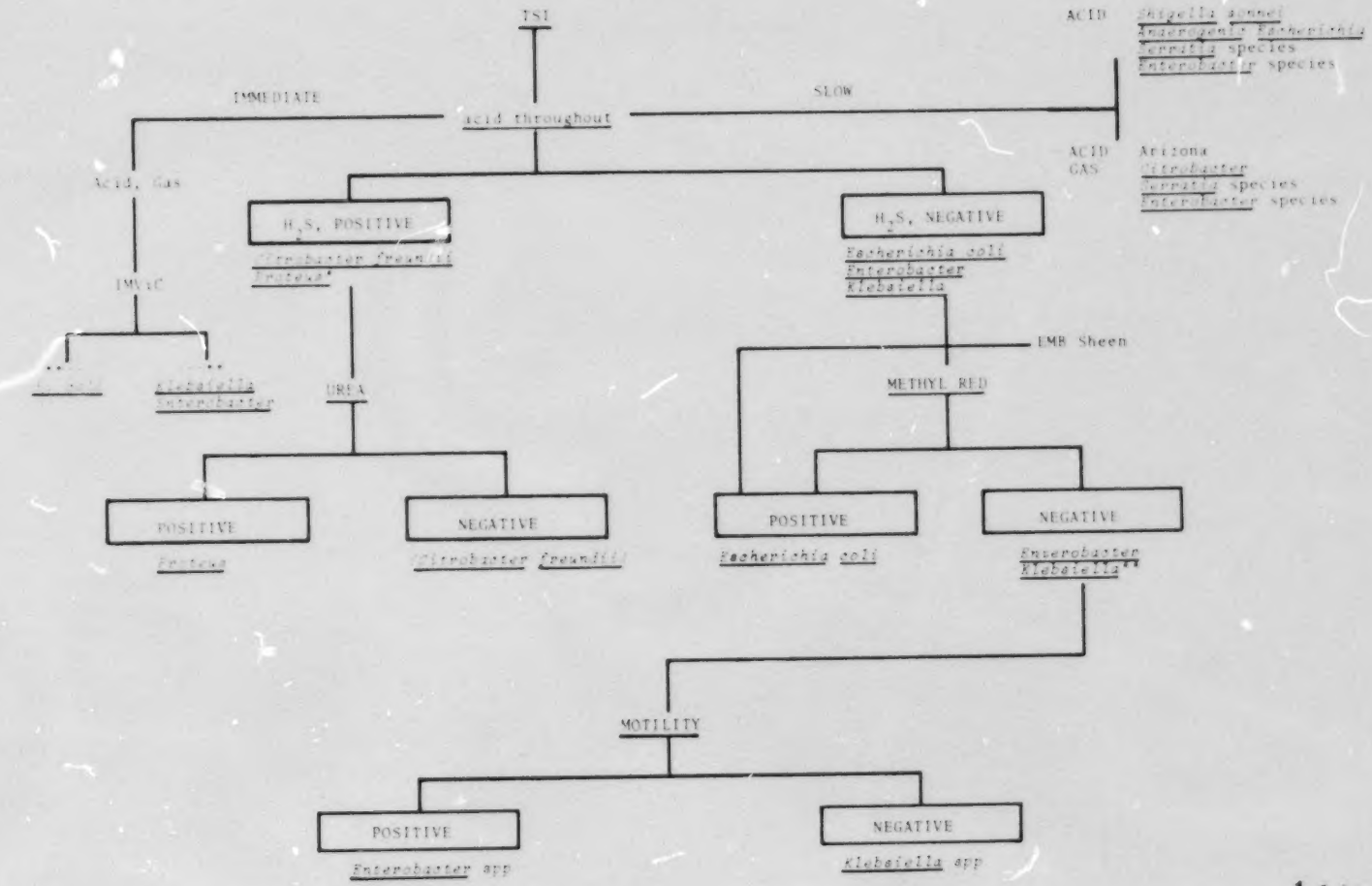
The methyl red test may be performed if identification of lactose fermenting organisms is desired.

b. Refer to chapter 14 for principles, techniques, and interpretations of the biochemical tests.

c. Considering the reactions of the above biochemical tests, figures 8-1 and 8-2 and table 8-1 will assist in the generic identification of enteric gram-negative bacilli.

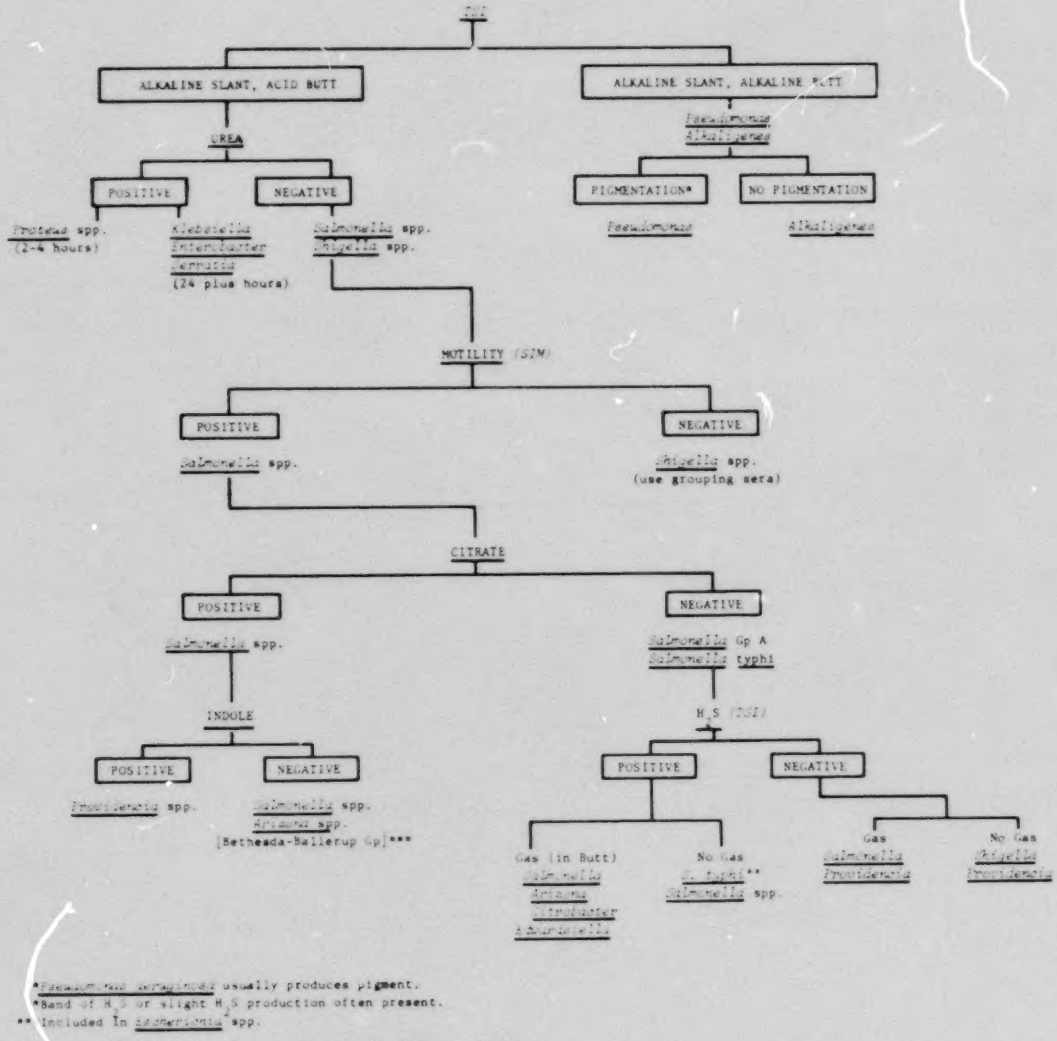
8-5. *Escherichia coli* (Escherich—1886):

*Delayed lactose fermenters will appear colorless at this stage.



*Certain strains ferment sucrose of TSI medium and produce acid slant and acid butt.
**Usually negative (includes former genus *Aerobacter*).

Figure 8–1. Lactose Fermenters (Flowchart).



*Pseudomonas aeruginosa usually produces pigment.
*Band of H_2S or slight H_2S production often present.
** Included In Escherichia spp.

Figure 8–2. Nonlactose Fermenters (Flowchart).

a. **Taxonomy:**

Part 8.
Family I—Enterobacteriaceae
Genus I—*Escherichia*
Species—*coli*

b. **Morphology and Staining.** *Escherichia coli* appear microscopically as short, plump rods measuring 0.4 to 0.7 μm in width and 1 to 4 μm in length. Coccoid forms are seen along with short chains in young cultures, or exudates from infected wounds. Motility among strains of *E. coli* may range from motile to sluggish to no motility at all. There are no sporeforming *E. coli* strains, but capsules may be produced by a small percentage of the known strains. The usual aniline dyes stain *E. coli* gram negative.

c. **Cultural Characteristics.** *Escherichia coli* is a facultative anaerobe growing readily on media such as blood agar, MacConkey's, EMB, and thioglycollate fluid at temperatures ranging between 20°C and 40°C. On agar plates, the surface colonies appear in 12 to 24 hours, reaching a size of 2 to 3 mm in 48 hours. Even though colonies may vary in individual appearance, on blood agar they typically grow to be low, convex, smooth, colorless to opaque colonies. They bunch together often, ranging in number from two colonies to several. Such arrangements can be seen by viewing plates 48 hours old or older.

d. **Reactions Used in Identification.** Many sugars are fermented by *E. coli:* glucose, lac-

Table 8–1. Biochemical Reactions of Enteric Bacteria.

	ESCHERICHIEAE		EDWARDSIELLEAE	SALMONELLEAE				KLEBSIELLEAE								PROTEAE					
						Citrobacter		*Klebsiella*	*Enterobacter*				*Serratia*			*Proteus*				*Providencia*	
	Escherichia	*Shigella*	*Edwardsiella*	*Salmonella*	*Arizona*	*freundii*	*diversus*	*pneumoniae*	*cloacae*	*aerogenes*	*hafniae*	*agglomerans*	*marcescens*	*liquefaciens*	*rubidaea*	*vulgaris*	*mirabilis*	*morganii*	*rettgeri*	*alcalifaciens*	*stuartii*
INDOLE	+	−ᵛ+	+	−	−	−	+	−	−	−	−	−ᵛ+	−	−	−	+	−	+	+	+	+
METHYL RED	+	+	+	+	+	+	+	−ᵛ+	−	−	−ᵛ+	−ᵛ+	−ᵛ+	+ ᵛ −	−ᵛ+	+	+	+	+	+	+
VOGES–PROSKAUER	−	−	−	−	−	−	−	+	+	+	+ᵛ−	+ᵛ−	+	−ᵛ+	+	−	−ᵛ+	−	−	−	−
SIMMONS CITRATE	−	−	−	d	+	+	+	+	+	+	d	d	+	+	+ᵛ(+)	d	+ᵛ(+)	−	+	+	+
HYDROGEN SULFIDE (TSI)	−	−	+	+	+	+ᵛ−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
UREASE	−	−	−	−	−	d[1]	d[1]	+	+ᵛ−	−	−	d[1]	d[1]	d[1]	d[1]	+	+	+	+	−	−
KCN	−	−	−	−	−	+	−	+	+	+	+	−ᵛ+	+	+	−ᵛ+	+	+	+	+	+	+
MOTILITY	+ᵛ−	−	+	+	+	+	+	−	+	+	+	+ᵛ−	+	+	+ᵛ−	+	+	+ᵛ−	+	+	+
GELATIN (22°C)	−	−	−	−	(+)	−	−	−	(+)ᵛ−	−ᵛ(+)	−	d	+ ᵛ (+)	+	+ᵛ (+)	+	+	−	−	−	−
LYSINE DECARBOXYLASE	d	−	+	+	+	−	−	+	−	+	+	−	+	+ ᵛ (+)	+ᵛ (+)	−	−	−	−	−	−
ARGININE DIHYDROLASE	d	d	−	+ᵛ (+)	+ᵛ(+)	d	+ᵛ(+)	−	+	−	d	−	−	−	−	−	−	−	−	−	−
ORNITHINE DECARBOXYLASE	d	d[2]	+	+	+	d	+	−	+	+	+	−	+	+	−	−	+	+	−	−	−
PHENYLALANINE DEAMINASE	−	−	−	−	−	−	−	−	−	−	−	−ᵛ+	−	−	−	+	+	+	+	+	+
MALONATE	−	−	−	−	+	−ᵛ+	+	+	+ᵛ−	+ᵛ−	+ᵛ−	+ᵛ−	−	−	+ᵛ−	−	−	−	−	−	−
GAS FROM GLUCOSE	+	−[3]	+	+	+	+	+	+	+	+	+	−ᵛ+	+ᵛ−[3]	+ᵛ−	d	+ᵛ−	+	+ᵛ−	−ᵛ+	+ᵛ−	−
LACTOSE	+	−[3]	−	−	d	(+)ᵛ+	d	+	+ᵛ(+)	+	d	d	−	d	+	−	−	−	−	−	−
SUCROSE	d	−[3]	−	−	−	d	−ᵛ+	+	+	+	d	d	+	+	+	+	d	−	d	d	(+)ᵛ+
MANNITOL	+	+ᵛ−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+ᵛ−	−	d
DULCITOL	d	d	−	d[2]	−	d	+ᵛ−	−ᵛ+	−ᵛ+	−	−	−ᵛ+	−	−	−	−	−	−	−	−	−

(1) Certain biotypes of *S. flexneri* produce gas, cultures of *S. sonnei* ferment lactose and sucrose slowly and decarboxylate ornithine.

(2) *S. typhi*, *S. choleraesuis*, *S. enteritidis* bioser. Paratyphi A and Pullorum, and a few others ordinarily do not ferment dulcitol promptly.

(3) Gas volumes produced by cultures of *Salmonella*, *Proteus*, and *Providencia* are small.

+, 90% or more positive in 1 or 2 days. −, 90% or more negative, d, different biochemical types [+, (+), −]. (+) delayed positive (decarboxylase reactions, 3 or 4 days). + or −, majority of cultures positive. − or +, majority negative. ᵛ, weakly positive reaction.

NB. This chart is simply a guide. Users are urged to consult other publications, such as CDC publications entitled "Biochemical Reactions Given by Enterobacteriaceae in Commonly Used Tests" and "Differentiation of Enterobacteriaceae by Biochemical Reactions" (W. H. Ewing, 1973), for percentage data, additional tests, and references.

Compiled by Dr. Wm. H. Ewing Enteric Bacteriology Laboratories DHEW-PHS-CDC, Atlanta, GA 30333 July 1973

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tose, and maltose, just to name a few. The fermentation process by *E. coli* produces acid and gas reactions. *Escherichia coli* gives positive reactions for the indole test and the methyl red test. Several species of Proteus give the same reaction on these two tests but can be differentiated on the basis of the TSI reaction.

e. **Serotyping.** Serotyping is a process by which further classification of *E. coli* is made possible. The organism possesses three antigens important to the serological classification of *E. coli*. These are: (1) the O or somatic (cellular) antigens which resist heat inactivation at 100°C for 1 hour; (2) the H or flagellar antigens which are inactivated by exposure to heat at 100°C for 1 hour; and (3) the K or enveloping cellular (capsular) antigens which surround the O antigens and prevent their agglutination in specific O antiserum. The K antigens are inactivated by exposing the organisms to heat at 100°C for 1 hour. To date, more than 148 antigenically distinct somatic O groups of *E. coli* have been identified. The K antigens of these groups are divisible into three varieties, designated L, A, and B. Collectively, these varieties account for a total of 91 distinct K antigens. Also, 49 H antigens are now known. Only 11 of the O antigenic groups have been incriminated as causative agents of enteric diseases, and all have been shown to possess the B variety of K antigen. The specific serotypes of *E. coli* associated with infant diarrhea are listed below:

O Antigen	*K Antigen*	*H Antigen*
O26	K60 (B6)	KM
O26	K60 (B6)	H11
O55	K59 (B5)	NM
O55	K59 (B5)	H6
O55	K59 (B5)	H7
O111a, 111b	K58 (B4)	NM
O111a, 111b	K58 (B4)	H2
O111a, 111b	K58 (B4)	H12
O125a, 125c	K70 (B15)	H21
O126	K71 (B16)	NM
O126	K71 (B16)	H27
O127a	K63 (B8)	NM
O128a, 128c	K67 (B12)	H12

Isolation of *E. coli* from infant diarrhea requires that the fecal material be inoculated to differential media (eosin methylene blue, MacConkey, or desoxycholate agar) rather than the more highly selective media used for isolation of *Salmonella* and *Shigella* spp; otherwise, growth will be inhibited. Blood agar should also be inoculated since occasional *E. coli* strains fail to develop on differential media. After 18 to 24 hours incubation, the plates are examined for typical colonies to *E. coli*.

f. **Agglutination Technique.** Since the colonies of enteropathogenic forms cannot be grossly distinguished with certainty from nonpathogenic forms, colonies should be tested serologically.

(1) *Subculturing Colonies:*

(a) Use ten or more colonies from blood-agar plates since differential or selective media may cause confusing results.

(b) Pick colonies from several sites on the culture plate.

(c) Subculture to a fresh blood agar plate, which has been marked off in six sections.

(d) Allow to grow overnight to increase the number of available test colonies.

(2) *Slide Agglutination Test—Testing With Pooled Antiserum:*

(a) This procedure may be used when time is at a premium.

(b) Draw an inoculating loop across a number of colonies and prepare an emulsion of the growth in a drop of saline on a glass slide.

(c) Mix a loopful of pooled (polyvalent) OB antiserum with the suspension. The antiserum pool contains specific antibodies against each of the O antigens and the B antigens of the 11 *E. coli* serotypes of infant diarrhea.

(d) Pronounced agglutination in the organism-antiserum mixture is presumptive evidence for the presence of an enteropathogenic *E. coli* serotype.

(3) *Serotyping:*

(a) Individual colonies in the OB pools are confirmed and subsequently tested with individual OB sera to identify the specific type.

(b) If the amount of growth present on the primary plates is insufficient, it may be necessary to subculture growth on a nutrient agar slant before preparing suspensions for typing in OB serums.

(c) Agglutination of a given serotype occurs rapidly in its specific OB antiserum. The reaction is primarily a result of B antigen combining with B antibody, and there is little or no cross reactivity with other OB serotypes.

(d) Once agglutination is obtained in one of the antisera, heat a heavy saline suspension of the organism for 1 hour in a water bath at 100°C. This will inactivate the thermolabile B (masking) antigen.

(e) Test the cooled suspension with the same OB antiserums previously employed for testing the unheated suspension. This should confirm the somatic O group to which the *E. coli* strain belongs.

(f) Both polyvalent OB antiserum and the individual OB antiserums for typing entero-

pathogenic *E. coli* strains are available through commerical sources.

g. **Bacteriophage Typing.** Based on antigenic serotype and biochemical testing, numerous strains of *E. coli* have appeared in the literature; however, in 1966 Brown and Parisi introduced the bacteriophage typing system which is easier and faster than serotyping. Bacteriophages are viral organisms specific to the host bacteria used to "phage type" the bacteria.

h. **Pathogenicity.** Although Escherichia coli is a normal intestinal flora in man, it can become pathogenic outside its usual habitat. When introduced into tissues, such as the urinary and respiratory tracts, or the peritoneum and meninges, serious and complicating infections can occur. This is also true of coliform infections of open wounds and ear or eye areas. *E. coli* is very common as a causative agent of systitis, an infection of the urinary tract. Certain strains of *E. coli* can produce a potent enterotoxin resembling the cholera toxin, capable of producing acute diarrhea without invasion of the intestinal epithelium. Pathogenic *E. coli* also causes diarrhea in children, particularly those under 2-years of age. This is the main reason for requiring "age of patient" on pediatric stool specimens submitted for culture, allowing the technician to determine whether or not *E. coli* colonies should be typed. Pure cultures of *E. coli* from adult stool may be indicative of the cause of diarrhea or intestinal distress. *E. coli* once rarely a causative agent in septicemias has increased in frequency. However, when normal host defenses are inadequate, particularly in early infancy, old age, in the terminal stages of other diseases, or after immuno-suppression, coliform bacteria may reach the bloodstream and cause sepsis.

i. **Growth Requirements.** No special requirements are necessary to obtain good growth of *Escherichia coli*. It can be cultured successfully on routine laboratory media (blood agar, MacConkey's EMB, etc).

j. **Identification.** *E. coli* is routinley differentiated from other enterics by plating suspected cultures on eosin methylene blue agar (EMB) and blood agar plates. *E. coli* ferments lactose, and colonies appear a characterisitc metallic green, giving a presumptive identification of the bacteria in question. Biochemicals may be used for identification of *E. coli* in all instances. From the TSI, *E. coli* is hydrogen sulfide (H_2S) negative, positive for indol (99.2 percent), methyl red positive, and negative for citrate utilization. After an enteric has been identified as *E. coli*, serotypes are performed to determine pathogenicity of the bacteria (section f). Presently 16 enteropathogenic *E. coli* serotypes are generally recognized. Polyvalent typing sera are routinely available in most laboratories for use in stool culture examinations.

k. **Key to Identification.** Colony characteristics of *E. coli* are metallic sheen on EMB and large, flat, nonviscous colonies.

8–6. ***Salmonella*** **(Salmon, 1885):**

a. **Taxonomy:**

Part 8.
Family—Enterobacteriaceae
Genus IV—*Salmonella*
Species—*Choleraesuis*
typhi
enteritidis

b. **Morphology and Staining.** Microscopically Salmonellae are gram-negative, nonsporeforming bacilli measuring 0.5 to 0.8 μm in width, and 1 to 3.5 μm in length. *Salmonella* may appear morphologically longer in old cultures, as well as in R-phase colonies. Most species are motile with flagella surrounding the bacterial cell (peritrichous). *Salmonella pullorum* and *S. gallinarum* are the only two species of the genus which are nonmotile.

c. **Cultural Characteristics.** *Salmonella* is a facultative anaerobe, growing readily on routinely used media. Incubation temperatures may range from 15°C to 41°C, with an optimum temperature of 35°C. The organism can be isolated directly from the blood or urine, and cultured on meat-infusion agar or broth, with the addition of bile, which reduces the bactericial effects of blood mechanisms. *S. typhi* colonies, following a 24-hour incubation, are smaller, more delicate, and more transparent than contemporary colonies. On media such as bismuth sulfite, they appear as jet black colonies. This characteristic is very helpful in isolation techniques revealing typhoid bacilli.

d. **Pathogenicity.** It is important to remember that all *Salmonellae* are pathogens and may produce enteric fever, septicemia, or gastroenteritis in man. Such infections often originate from ingestion of contaminated food or drink. The enteric fevers consist of typhoid and paratyphoid fever. *Salmonella typhi* is responsible for typhoid, while *S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, and *S. sendai* are most often encountered in paratyphoid fever.

(1) *Enteric Fevers.* In enteric fevers, the ingested organisms enter the small intestine, spread through the intestinal lymphatics to the thoracic duct, and enter the blood stream.

The resultant septicemia distributes the infection to many organs including the kidneys, intestines, liver, gallbladder, and other tissues. Infections are characterized by an insidious onset, with low grade fever that ultimately becomes quite high during the bacteremic phase. Blood cultures are usually positive only during the first and second weeks of infection. Stool and urine cultures usually fail to yield the responsible *Salmonella* spp until the third week. The duration of typhoid and paratyphoid fever is usually several weeks.

(2) *Septicemia.* Salmonellae infections that result in septicemia are often due to *Salmonella cholerae-suis, Salmonella typhi* or *Salmonella enteritidis* serotypes *paratyphi* A and B. The onset of symptoms is abrupt since bloodstream invasion occurs within a short period of time following oral ingestion of the bacteria. This is accompanied by a rapid rise in temperature that spikes during the height of infection. Wide distribution of the organism results in focal suppuration and abscess formation in various tissues. Meningitis, osteomyelitis, endocarditis, and pneumonia are known complications of such infections. Blood cultures are most often positive when taken during the height of fever.

(3) *Gastroenteritis.* Of the many *Salmonella* spp which produce acute gastroenteritis in man, *S. typhimurium* is the most frequent causative agent. *Salmonella enteritidis* is possibly the second most common cause. *S. cholerae suis* has also been implicated in gastroenteritis to a much lesser extent than either of the above two species. Infections are characterized by fairly sudden onset (15 to 24 hours incubation), and rather severe gastrointestinal distress with vomiting, diarrhea, and slight elevation of temperature. Recovery is rapid (1 to 3 days) since the intestinal tract is not usually invaded by the organisms. Symptoms result from the irritative action of acids and endotoxins upon the intestinal mucosa. The acids are formed by fermentation of carbohydrates by the responsible organisms. Endotoxins which are composed of the lipopolysaccharides of the cell wall are released following death and cellular lysis of the etiologic agent. Only very rarely do infections develop into septicemia. Outbreaks of gastroenteritis are usually linked with the consumption of certain foods and are often referred to as "food poisoning." Diseases usually originate from unsuspected subclinical cases, convalescent carriers, or healthy permanent carriers who harbor the organisms in their intestines, gallbladders, or urinary tracts. Such individuals may contaminate food or drink either directly or indirectly. The Salmonella produce no exotoxins. Upon death and lysis of the cells, endotoxins are released which largely account for the disease symptoms of man.

e. **Identification:**

(1) *Specimen Culture:*

(a) In the first stages of the disease it is best to perform blood cultures, rather than stool cultures, since the organism does not at first appear in the stool.

(b) In about a week or 8 days, the organism will not be found in the blood, but in the stool.

(c) At onset stool cultures may be negative.

(d) In about 25 percent of typhoid cases, urine cultures are positive. Centrifuging urine is recommended prior to culturing to concentrate any bacteria before plating the media.

(2) *Somatic "O" Agglutination:*

(a) Use slide agglutination test to identify possible *Salmonella* spp.

(b) Prepare a dense, homogenous saline suspension of the organism from the TSI slant.

(c) An approximate 3 mm loopful of this suspension should be reacted with an equal amount of antiserum.

(d) Test first with polyvalent antiserum which contains antibodies against Salmonella somatic group antigens A through E.

(e) Complete and immediate agglutination will be observed with most Salmonellae. A few of the paracolons and some Shigella may exhibit partial to complete agglutination.

(f) Test those cultures agglutinated by polyvalent antiserum with individual grouping antiserums A though E. The particular serum in which clumping occurs most rapidly is the serological group to which the organism belongs.

(g) Although cross-reactions sometimes occur between various O groups of the Salmonellae, such reactions are not usually pronounced.

(h) Report any culture which exhibits prompt, definite clumping in polyvalent serum and in one or more of the O grouping serums as a probable *Salmonella* sp.

(i) Biochemical confirmation is necessary, since many paracolon bacteria contain antigens related to the O antigens of *Salmonella* spp.

(3) *"Vi" Antigen Agglutination:*

(a) If a culture fails to react in polyvalent antiserum, it should be tested with Vi antiserum. This antiserum will detect the presence of Vi antigen which is present on the cell

surface of most freshly isolated strains of *typhi* and occasional *S. enteritidis* serotype paratyphi C organisms.

(b) If agglutination occurs with Vi antiserum, heat the saline suspension in a boiling bath for 15 minutes.

(c) Cool the suspension and retest with polyvalent O. Heating removes the Vi (masking) antigen from the cell surface, permitting the O antibodies to react with somatic antigens to produce agglutination.

(d) Once the presence of Vi antigen has been established and agglutination observed in polyvalent antiserum, test the previously boiled cells with anti-D or anti-C group serums. The particular antiserum used is determined by the preliminary biochemical reactions previously observed.

(e) Possible *typhi* strains should agglutinate in D group antiserum, and *S. enteritidis* serotype *paratyphi* C cultures in C antiserum.

(f) Cultures which react with Vi antiserum and which, after boiling, are agglutinated with group D or group C antiserum may be safely assumed to be strains of *typhi* or *S. enteritidis* serotype paratyphi C.

(g) All cultures which fail to react quickly and strongly with polyvalent or Vi antisera and cultures which react with polyvalent antiserum but fail to react typically with O grouping antiserums should be subjected to further biochemical tests.

(h) Potassium cyanide medium is of great value in eliminating *Citrobacter* spp, which can resemble the Salmonellae. *Citrobacter* spp grow in potassium cyanide (KCN) broth, while all Salmonellae fail to do so.

(4) *"H" Antigen Agglutination.* Once the O group of a *Salmonella* culture has been determined, species definition is accomplished by subjecting the organism to H (flagellar) antiserums and subsequent biochemical testing.

(a) Test the unheated suspension of the organism on a slide with H antiserums, which are diluted 1:50 or 1:100, using the same technique as for O agglutination.

(b) Another acceptable technique is to dilute a broth culture of the test organism equally with normal saline containing 0.6 percent formalin. React this with appropriate antiserums at 50°C. Dilute the antiserums for this procedure 1:1000. Read the results following 1 hour of incubation in a water bath. The technique employed will be determined by the specific directions accompanying various lots of antiserums procured.

(c) *Group A* organisms should be tested with *a* antiserum. Agglutination assures that these cultures are *Salmonella enteritidis* bioserotype *paratyphi* A (see table 8–2).

(d) *Group B* organisms should be tested with H (flagellar) antiserums which react with *b* or *i* antiserums and 1, 2, 3, and 5 phase 2 antigens. Sometimes Group B cultures will be agglutinated by 1, 2, 3, and 5 antiserum (against phase 2 antigens), yet fail to react with *b* or *i* antiserums (against phase 1 antigens). Experience has shown that freshly isolated diphasic strains frequently occur in only one phase. Therefore, if agglutination tests reveal that only phase 2 is present, the organism must be recultivated in an environment which will induce phase 1 development. This is necessary because phase 1 flagellar antigens must be present if culture identity is to be established. Such cultures are inoculated into a tube containing 2 to 3 ml of semisolid infusion medium to which one loopful of 1, 2, 3, and 5 antiserum was previously added and mixed just before solidification. After 18 to 24 hours incubation, phase 1 organisms may be picked from the spreading growth present throughout the medium. Once phase 1 antigens are obtained, the organism is again exposed to *b* and *i* serums to determine whether it is *S. enteritidis, S. paratyphi-B,* or *S. typhimurium* (see table 8–2).

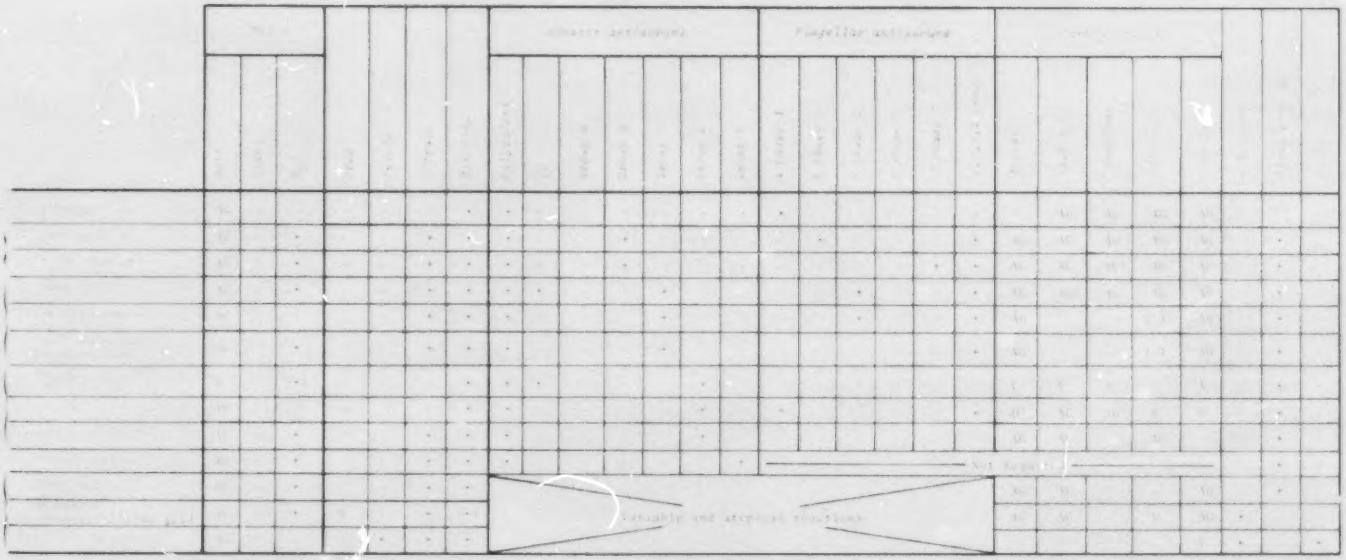
(e) *Group C* organisms should be tested with *c* (phase 1) and 1, 3, 4, and 5 (phase 2) antiserums. If agglutination occurs only in 1, 2, 3, and 5 (phase 2) antiserum, then the organism must be induced to form phase 1 flagellar antigens using the same procedure as described for Group B cultures. Once phase 1 is obtained, the organism is retested in *c* antiserum. Since most strains of *Salmonella choleraesuis* belong to the serotype Kunzendorf, wherein phase 1 antigens are generally suppressed, the organism may be identified without phase 1 typing (see table 8–3).

(f) *Group D* organisms should be checked for agglutination in *a, d,* and 1, 2, 3, and 5 antiserums. The presence of Vi antigen and the agglutination of heat-treated cells in group D somatic antiserum are usually sufficient features for confirming strains of *S. typhi* that are biochemically typical (see table 8–2).

(g) *Group E* organisms possess such a diversity of flagellar antigens that no further examination is routinely accomplished. These forms are simply reported as *Salmonella* spp, Group E.

(h) The Kauffman-White schema lists the serologic groups of the genus *Salmonella*

Table 8-2. Biochemical and Serological Reactions of the Salmonellae.



derived on the basis of somatic antigens possessed by the various strains. Each group is further subdivided into serologic types in accordance with the distribution of phase 1 and phase 2 antigens. This classification system (Edwards and others, 1965) greatly simplifies the serological identification of these forms. Table 8–2 summarizes the salient biochemical and serological reactions for identification of the *Salmonellae* most often responsible for human infections. The table also includes reactions for the genus *Arizona*, Bethesda-Ballerup (now included under the genus *Citrobacter*), and genus *Providencia* since these forms are often confused with *Salmonella* spp. Of particular importance are the reactions of these organisms with potassium cyanide broth and the ninhydrin and ferric chloride tests. Collectively, these reactions not only enable differentiation of paracolons from *Salmonellae*, but they also allow separation of the individual paracolon groups. The carbohydrate, dulcitol, is also useful in this connection. Those laboratories which do not perform grouping and typing should tentatively identify the organisms biochemically and send such cultures to a reference laboratory for confirmation.

f. **Growth Requirements.** There are no special growth requirements for *Salmonella*. Instead, some choice media may be mentioned. Media such as Salmonella-Shigella (SS), bismuth sulfite, eosin-methylene-blue (EMB), MacConkey agar, xylose lysine desoxycholate agar (XLD), and HE (Hektoen media) are excellent for isolating the typhoid bacilli because lactose is not fermented. Bacteria which ferment lactose of the aforementioned media change the pH thereby giving characteristic color to the bacterial colonies. *Salmonella* is colorless on all media mentioned except when a rare strain may attack lactose and bismuth sulfite, on which it is black. (See figures 8–1 and 8–2 for flowcharts for lactose and nonlactose fermenters.)

8–7. **Genus *Arizona*.** These organisms are biochemically and serologically related to the *Salmonella*. Although they are often mistaken for *Salmonella* spp, the consequences of such misdiagnoses are not serious since the organisms produce clinically similar diseases and respond to the same therapeutic measures. *A. hinshawii* has been principally isolated from the intestinal contents of reptiles and to a lesser degree from stools of humans. Some texts refer to Arizona as a group of paracolon bacilli which can be a severe pathogen in man; others prefer to place it in a genus alone under the genus Salmonella because of similarities. Biochemical reactions are given in table 8–2.

8–8. ***Shigella* (Shiga, 1898):**

a. **Taxonomy:**

Family—Enterobacteriaceae
Genus V—*Shigella*
Species— *dysenteriae*
flexneri
boydii
sonnei

b. **Morphology.** Morphologically, Shigella bacilli are short rods measuring 0.5 μm to 0.7 μm in width and 2 to 3 μm in length. They are not encapsulated and do not form spores. *Shigella* is nonmotile and stains gram-negative with the usual aniline dyes.

c. **Cultural Characteristics.** *Shigella* is a facultative anaerobe, growing readily on routinely used laboratory media. Temperatures for good growth range from 10°C to 40°C. The usual optimum for bacteria is 35°C, but species such as *S. flexneri* and *S. sonnei* grow at 45°C (media pH 6.4 to 7.8). After 24 hours incubation the bacilli appear as 2-mm, circular, convex colonies, colorless (whitish-clear) with a smooth surface. Unlike *Salmonella*, *Shigella* grows poorly on either bismuth sulfite or brilliant green agar media.

d. **Pathogenicity.** The Shigella may cause bacillary dysentery. Infections are usually limited to the gastrointestinal tract. Three species of the genus *Shigella* are usually isolated from such cases: *S. dysenteriae, S. flexneri,* and *S. sonnei. S. flexneri* and *S. sonnei* are the most frequently isolated organisms in this country, while *S. dysenteriae* are relatively frequent in India, Japan, China, and other parts of Asia. The disease process is essentially an inflammation of the mucous membrance of the large intestine and terminal ileum which leads to necrosis and superficial ulceration. Symptoms occur within 1 to 2 days following ingestion of contaminated food or drink. The illness is characterized by sudden onset of abdominal pains, cramps, diarrhea, and fever. The intense irritation of the bowel is due to the release of somatic endotoxin upon autolysis of the *Shigella* spp. Infections from *S. dysenteriae*, type 1, are more severe because, in addition to the endotoxin substance, an exotoxin (neurotoxin) is produced which causes paralytic symptoms. Although some individuals recover quickly from bacillary dysentery and pass infectious bacilli in stools for only a short period, others become chronic carriers (ulcerative colitis) and may

suffer frequent relapses of the disease. The latter serve as a reservoir of infection.

e. **Growth Requirements.** As mentioned previously, the *Shigella* bacilli do not grow as well on the selective media, bismuth sulfite and brilliant green agar, as do the *Salmonella* bacilli. However, *Salmonella-Shigella*, EMB, XLD, HE, or MacConkey's media are routinely used to recover *Shigella* from stool specimens for diagnosis. (S-S grows only 30 percent of Shigella isolates.)

f. **Identification.** Biochemically the Shigella ferment glucose, and some ferment mannitol. They do not produce gas when inoculated into sugar tubes (except for *S. flexneri*, type 6), Generally, they do not cause change on any of the usual biochemicals utilized (for example, citrate, lysine, lactose, glucose, or arginine), as can be seen in table 8-3. There are serotypes within this genus, that are similar to those of *Escherichia coli* and require biochemical tests to differentiate the *Shigella*. This similarity is the reason that the genus *Shigella* is placed in the "tribe" Escherichieae, in the older nomenclature. The tribe Escherichieae is no longer recognized under the newer taxonomy (1974).

(1) *Slide Agglutination.* Stool isolates suspected of being *Shigella* (A thru D) subgroup (see table 8-3), should be tested with the individual polyvalent *Shigella* grouping antiserums. Four antiserums are recommended for *Shigella* spp, each containing antibodies specific for members of *Shigella* subgroups A, B, C, and D. More specifically, these antiserums will agglutinate the serotypes of each *Shigella* subgroup or species as indicated below. By employing the five antiserums listed in table 8-3, it is possible to group all but a few of the rarer *Shigella*. Slide agglutination tests are generally used in the serological identification of these organisms. A heavy suspension of the organism to be tested is prepared by emulsifying growth from an 18 to 24 hour slant culture in 0.5 ml of physiological saline. Pure culture growth from TSI slants may be used for this purpose or infusion agar slants may be inoculated and incubated specifically for such examinations. Specific agglutination usually takes place within 1 minute with most commercially prepared antiserums. The saline and normal serum controls should reveal no clumping. Delayed or incomplete cross reactions are significant. Such cross reactions are due to antigenic similarities between various *Shigella* subgroups. It is important to remember that other Enterobacteriaceae occasionally encountered possess somatic O antigens related to those of the *Shigella*. For this reason, cultural and biochemical studies, as well as serological procedures should be employed for identification of suspected cultures. It is imperative that the directions accompanying various commercially pre-

Table 8-3. Biochemical and Serological Reactions of the Shigellae.

	TSI			Urea	Indole	Citrate	Motility	Polyvalent O antiserums					Carbohydrates					
	Butt	Slant	H_2S					Subgroup A	Subgroup B	Subgroup C	Subgroup D	A-D Group	Lactose	Mannitol	Xylose	Rhamnose	Salicin	Sorbitol
Shigella dysenteriae (A)	A	-	-	-	(-)	-	-	+	-	-	-	-	-	-	-	(-)	-	(-)
Shigella flexneri (B)	A	-	-	-	(+)	-	-	-	+	-	-	-	-	A	-	(-)	(-)	(-)
Shigella boydii (C)	A	-	-	-	(-)	-	-	-	-	+	-	-	-	A	(A)	(-)	(-)	A
Shigella sonnei (D)	A	(-)	-	-	-	-	-	-	-	-	+	-	(D)	A	-	A	-	-
Alkalescens-Dispar (A-D)	A	V	-	-	+	-	-	-	-	-	-	+	(D)	A	A	(A)	V	(A)

KEY:

A = Acid

D = Delayed acid formation--more than 24 hours

+ = Reaction or production

- = Nonreactive or no change

(-) = Usual reaction, occasional strains to contrary

V = Some strains positive, some negative

*Included under genus *Salmonella*.

NOTE: 1. TSI, citrate and motility media are read after 18-24 hours incubation; indol and carbohydrates are checked between 24-48 hours incubation.

2. Biochemically typical *Shigella* and members of the A-D Group* often possess enveloping antigens and may, therefore, be nonreactive in grouping antiserums. Such strains must be heated and then retested (see *Shigella* identification) with grouping serums.

pared antiserums be followed explicitly if slide agglutination results are to be relied upon. If an organism appears to be *Shigella* spp, yet fails to agglutinate in any of the antiserums, the saline suspension should be heated in a boiling water bath for 30 minutes and then retested. Many *Shigella* cultures possess envelope surface antigens which prevent the somatic antiserums from coming in contact with somatic antigens. Therefore, specific agglutination is impossible unless the surface antigen is first inactivated by heat. Members of the A-D group also contain enveloping antigens and thus require similar treatment. When a suspected *Shigella* culture still fails to react with *Shigella* grouping serums, even after heating, the unheated organism should be tested with *Salmonella* polyvalent and Vi antiserums (see identification of the *Salmonella*, this chapter). This is necessary because certain nonmotile *Salmonella typhi* strains which fail to produce hydrogen sulfide (18–24 hours) resemble the *Shigella* on triple sugar iron agar slants. Other atypical *Salmonella* are occasionally encountered which resemble *Shigella* spp upon tentative biochemical and cultural analyses. Laboratories which do not perform *Shigella* grouping should tentatively identify the organisms biochemically and send such cultures to a reference laboratory for confirmation. All serologically typical *Shigella* cultures should also be subjected to biochemical tests for confirmation. Table 8–3 may be consulted for the serological and biochemical identification of *Shigella* subgroups and members of the "Alkalescens-Dispar" group (Escherichia) encountered in man. Of particular importance are the various carbohydrates listed in the table. They may be used to determine the probable species or *Shigella* subgroup in lieu of serological agglutination tests.

(2) *Biochemical Test.* (See paragraph 8–4(g).)

g. **Key for Identification.** Identification characteristics of *Shigella* are nonfermentation of lactose, nonmotility, and nonproduction of gas from dextrose.

8–9. The Alkalescens-Dispar Group. Although these organisms were formerly included in the genus *Shigella* as *S. alkalescens* and *S. dispar*, they are rarely, if ever, associated with enteric infections of man. Furthermore, *Alkalescens-Dispar* strains are now included in the genus *Escherichia*, since a strong biochemical and serological relationship exists between the two groups. Alkalescens-Dispar under *Escherichia* are regarded as two separate species, *E. alkalescens* and *E. dispar;* however, consensus is toward making the two a group of enteric bacilli distinct from Shigella and other closely related coliform bacilli. To date, authorities feel there is not enough justification to separate Alkalescens-Dispar as a genus or species, and it is therefore included in the genus *Escherichia.*

8–10. Saprophytic Enteric Flora:

a. **Taxonomy:**

Part 8.
Family I—Enterobacteriaceae
Genus II—*Edwardsiella*
Species—*tarda*
Genus VI—*Klebsiella*
Species—*pneumoniae*
ozaenae
rhinoscleromatis
Genus VII—Enterobacter
Species—*cloacae*
aerogenes
liquefaciens
Genus VIII—*Hafnia*
Genus—Pectobacterium (under Genus XII Erwinia)
Species—*carotovorum*
Genus—Serratia
Genus X—Proteus
Species—*vulgaris*
mirabilis
morganii
rettgeri
Genus II—Providencia
Species—*alcalifaciens*
stuartii
Genus—*Alcaligenes* (Part 7—Genera of uncertain affiliation)
Species—*faecalis*
Family I—Pseudomonadaceae
Genus I—*Pseudomonas*
Species—*aeruginosa*
pseudomallei
maltophilia

b. **Morphology.** Morphologically these genera cannot be distinguished from each other. Hence, size and gram staining are only useful when a nonselective or differentiating media is used.

c. **Cultural Characteristics.** Variations in the cultural characteristics of the so-called normal flora of feces are interesting and in older cultures are very characteristic, as in the case of *Proteus vulgaris* and *P. mirabilis*, which swarm over media such as blood agar. *Klebsiella* exhibits a stringing or very mucoid appearance in some species (*Klebsiella pneumonia*), while it may also be confused with late lactose-

fermenting *E. coli* or *E. freundii*, which is now placed under the genus *Citrobacter* (*C. freundii*). These groups of bacteria only resemble each other in the early stages of incubation, but since early diagnosis is important, biochemicals are sued primarily to save time. For example, if the urea tube on a suspected *Proteus* is negative, further testing is necessary, requiring an additional day's wait for the patient or physician.

d. **Pathogenicity.** Members of the general *Klebsiella*, *Enterobacter*, *Proteus*, and *Providencia Pseudomonas* and *Alcaligenes* are discarded as nonpathogenic in the adult intestine, but any organism can become a pathogen depending on the site of infection and the physical condition of the host.

(1) *Klebsiella* and *Enterobacter*. *Klebsiella pneumoniae* (Friedlander's bacillus) is isolated with some frequency from the upper respiratory and intestinal tracts of normal individuals and is responsible for a number of the bacterial pneumonias. Pulmonary infections are characterized by extensive hemorrhagi cconsolidation of the lobes. The fatality rate is high in untreated cases. *Klebsiella* spp are frequently isolated from various upper respiratory tract infections, although their presence, in many instances, is probably that of secondary invaders. The organisms have definite been responsible for suppurative abscesses of the liver and other visceral tissue. The pathogenic nature of *Enterobacter* is similar to that of *Escherichia*.

(2) *Proteus and Providencia Species*. Of the genus *Proteus*, four species are recognized: *Proteus vulgaris*, *P. mirabilis*, *P. morganii*, and *P. rettgeri*. Although these organisms are primarily free-living in water, soil, and sewage, they are frequently isolated from fecal specimens of normal individuals. *Proteus morganii* has been responsible for "summer diarrhea" of infants and children. *Proteus* spp rarely cause human infections, except when introduced into tissues other than the normal intestinal tract. In this connection, *Proteus* spp rank next to *E. coli* as the etiological agent of cystitis. The organisms are encountered frequently in eye and ear infections and occasionally in pleurisy, peritonitis, and suppurative abscesses in many areas of the body. *Proteus* spp are commonly associated with other bacteria in purulent wounds and may contribute to the severity of such infections. *Providencia alcalifaciens* and *P. stuartii* have been included in the genus Proteus due to sufficient biochemical similarities and may often require differentiation from *Proteus* species *P. maorganii* and *P. rettgeri*. (*Providencia* has been included as a species of the genus *Proteus* by some authors, as *P. inconstans*, allowing for a single species.)

(3) *Pseudomonas Species*. *Pseudomonas* spp occur widely in their natural habitats of soil, water, sewage, and air. *Pseudomonas aeruginosa* is often present in the normal intestine where it is rarely pathogenic. In other body areas, *P. aeruginosa* may produce severe infection. Infections of the ear (otitis externa, otitis media, and mastoiditis) are most often due to *P. aeruginosa*. The organism may infect surgical wounds, severe burns, and other injuries, causing the formation of blue-green pus. Infections of the eye and urinary tract are also encountered. It has been responsible for abscesses of the peritoneum, septicemis, endocarditis, and meningitis. *Pseudomonas aeruginosa* may invade the tissues of debilitated persons or infants.

(4) *Alcaligenes Species*. *Alcaligenes faecalis* is rarely pathogenic. Although strains of the organism have been found in cases of bacteremia, meningitis, and infections of the urinary tract, such instances are rare.

e. **Growth Requirements.** The normal flora of stools grow well on most media and require no special considerations as to content.

f. **Key for Identification.** Consult table 8–4 for comparative identification of general mentioned in this section.

8–11. *Vibrio cholera* (Robert Koch, 1883):

a. **Taxonomy:**

Family II—Vibrionaceae (Part 8)
Genus I—*Vibrio*
Species—*cholera*, 1883
Bio-type—El Tor, 1905

b. **Morphology.** Vibrios are comma-shaped, curved rods that measure 0.2 to 0.4 mm in width and 2 to 4 μm in length, and possess a single polar flagellum. The number of curved forms in the organisms varies; in older cultures, there may be very few curved forms.

c. **Cultural Characteristics.** *V. cholera* is aerobic and grows anaerobically with difficulty. It is motile and ferments glucose, galactose, maltose, and sucrose, producing no gas. It liquefies gelatin in 18 to 24 hours, routinely. After 18 to 24 hours of incubation (aerobic) at 35°C, colonies are round, slightly convex, translucent, and grayish-yellow in appearance. On blood agar, colonies are usually 1 to 2 mm in diameter, exhibiting a surrounding greenish zone. On MacConkey's agar, colonies are smaller due to inhibitors, and produce colorless

Table 8–4. Biochemical Reaction of Normal (Saprophytic) Enteric Flora.

	TSI			IMViC							Carbohydrate Fermentations							
	Butt	Slant	H2S	Indole	MR	VP	Citrate	Urea	Motility	Gelatin	Glucose	Lactose	Sucrose	Mannitol	Maltose	Adonitol	Salicin	Xylose
Salmonella sp.	AG	A	-	+	+	-	-	-	V	-	AG	AG	-	AG	AG	-	V	AG
Citrobacter freundii	AG	A	+	(-)	+	-	+	V	+	-	AG	AG	V	AG	AG	-	V	AG
Enterobacter cloacae	AG	(A)	-	V	+	+	+	-	+	+	AG	AG	AG	AG	AG	V	AG	V
Klebsiella pneumoniae	AG	(A)	+	-	-	(+)	(+)	D	-	-	AG	AG	AG	AG	V	AG	AG	AG
Proteus vulgaris	AG	V	+	+	+	-	-	+	+	+	AG	-	AG	-	AG	-	AG	AG
Proteus mirabilis	AG	V	-	-	-	+	+	+	+	+	AG	-	AG	-	-	-	-	AG
Proteus morganii	AG	(-)	-	+	+	-	-	+	+	-	AG	-	(-)	-	-	-	-	-
Proteus rettgeri	A	-	-	+	+	-	+	+	(+)	-	A	-	A	A		A	A	-
Alcaligenes fecalis	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	+	-	+	-	(-)	-	-	-	-	-	-	-

KEY:

A = Acid

D = Delayed acid formation--more than 48 hours

+ = Reaction or production

- = Nonreactive or no change

( ) = Usual reaction, occasional strains to contrary

V = Some strains positive, some negative

NOTE: 1. *C. Freundii* strains may hydrolyze urea slowly, (24-48 hours incubation) or not at all. *Klebsiella pneumoniae* will typically hydrolyze urea causing slightly positive reactions--18-24 hours. *Proteus* spp. usually exhibit complete urea hydrolysis within 2-4 hours incubation.

2. TSI, citrate, motility, and gelatin are read between 18-24 hours incubation. Indole, VP and carbohydrates are read between 24-48 hours while MR usually requires 3-4 days.

colonies. *V. cholera* grows very well in bile salt agar or peptone water (appearing as a pellicle on the surface). Thiosulfate citrate bile salt sucrose agar (TCBS) colonies grow with a distinct yellow pigment, with an opaque effect under transmitted light. TCBS requires no sterilization; hence, it is easy to use as well as handy. *V. cholera* thrives at a pH of 7.8 to 9.0.

d. **Pathogenicity.** Since 1817, cholera has been endemic or pandemic in areas such as West Bengal, East Pakistan, and portions of Southeast Asia, killing thousands of inhabitants. *V. cholera* (*V. comma*) is the etiologic agent of cholera in man. In cases of cholera, the *Vibrios* do not invade the blood stream or other tissues, but remain confined within the intestinal tract where they multiply rapidly and release endotoxin upon lysis of the cells. The endotoxin is extremely irritating to the intestinal mucosa and destroys epithelial cells. This causes vomiting and profuse diarrhea, resulting in severe dehydration, hypochloremia, acidosis, circulatory failure, and anuria. The loss of essential electrolytes is followed by hypovolemic shock; metabolic acidosis will result in death if the patient is not treated immediately. The mortality rate ranges between 30 and 50 percent. Cholera is transmitted from infected individuals or convalescing carriers through food, water, or by direct contact.

e. **Growth Requirements.** The presence of "bile salts" in media used to grow *V. cholera* is advantageous, but not a definite requirement for growth. However, the maintenance of the pH range 7.8 to 9.0 is important for successful growth of the organism.

f. **Identification.** In instances where cholera is suspected, fecal material, preferably the mucous flecks (exhibiting *Vibrios* in gram-stained smears) therein, are picked with a wire loop and streaked on plates of blood agar, nutrient agar, and MacConkey's agar. In addition, particulate matter or a few drops of stool should be inoculated to a tube of 1.0 percent

peptone water. Gohar broth also may be used to good advantage since the medium is inhibitory to gram-negative organisms other than *V. cholerae*. These media should have a pH of 7.8 to 9.0; otherwise, growth will be sparse or lacking. After 18 to 24 hours of aerobic incubation at 35°C, typical colonies of *V. cholerae* are round, slightly convex, translucent, grayish-yellow, smooth, and granular in transmitted light. On blood agar, the colonies are usually 1 to 2 mm in diameter and exhibit a greenish zone. On MacConkey agar, the colonies are usually smaller and produce colorless colonies. Growth of *V. cholerae* is more rapid (6 to 8 hours) in peptone water than is growth of other enteric bacilli, and is observed as a surface pellicle. Gram-stained smears of the primary growth (colonies or pellicle) will reveal distinctly curved gram-negative rods with rounded ends. In most instances, one end of a rod will appear more blunt than the other. This gives the organisms the shape of a "comma." The cells may be arranged singly, in S-shaped pairs, or rarely in short spiraled chains. At this point, it may be necessary to subculture the pellicle from peptone water to blood, nutrient, and MacConkey agars when growth is not exhibited on the primary streak plates. After typical growth suggestive of *V. cholerae* is obtained, pure isolates are subjected to the studies summarized in table 8–5. CAUTION: *V. cholerae* is an extremely dangerous pathogen, and despite its not forming spores, may be quite resistant to an adverse environment. Strict aseptic technique should be employed; a bacteriological hood may be used to advantage.

(1) *Biochemical Testing* (*V. cholera*):

(a) Perform a motility test (*V. cholera* is actively motile).

(b) Inoculate 1 percent tryptophan broth containing 0.001 percent potassium nitrate (final pH 7.8 to 8.0). After 24 hours incubation, a few drops of commercial grade sulfuric acid are added to the culture. If indole is produced and nitrates are reduced, a deep rose color will develop rather quickly. This is the "cholera-red" reaction. Although *V. chlorea* is not the only enteric organism which will exhibit the cholera-red reaction, a positive result is presumptive.

(c) Inoculate one tube each of phenol broth base containing 1 percent glucose, galactose, maltose, sucrose, mannitol, lactose, and dulcitol. After 24 to 48 hours incubation, *Vibrio cholera* will exhibit the reactions shown in table 8–5.

(d) Perform the hemolysin test. This is accomplished by mixing 0.2 ml of a 5 percent saline suspension of washed sheep erythrocytes with an equal quantity of 24-hour broth culture of the *Vibrio* under investigation. The mixture is incubated for 2 hours at 35°C, refrigerated overnight, and then examined for hemolysis. *Vibrio cholera* is nonhemolytic. Other *Vibrios* are hemolytic. Although colonies of *V. cholera* exhibit zones of greenish discoloration on blood agar, this is due to a hemodigestive process rather than secretion of true hemolysin.

Table 8–5. Cultural Characteristics of *Vibrio cholerae*.

Test	Reaction
Motility	Positive.
Cholera-red reaction (Nitroso-Indol)	Positive.
Carbohydrate fermentation (acid, no gas).	Glucose, galactose, maltose, sucrose, and mannitol-positive in 1–5 days. Lactose and dulcitol-negative.
Hemolysis (tube method).	Nonhemolytic.
Hydrogen sulfide production.	Positive, 18–24 hours.
Gelatin liquefaction	Positive, 18–24 hours.

(e) Inoculate SIM medium for the determination of hydrogen sulfide production after 18 to 24 hours incubation. This is evidenced by blackening of the medium.

(f) Inoculate a tube of gelatin and observe for liquefaction after 18 to 24 hours incubation.

(2) *Serological Identification.* All *Vibrio* isolates which morphologically, culturally, and biochemically appear to be *V. cholera* may be identified serologically. All motile *Vibrios* possess heat-labile H (flagellar) antigens, which do not contribute to serological identification. The various strains of *Vibrios* also contain heat-stable O (somatic) antigens, each of which confers serologic specificity upon injection into laboratory animals. On the basis of antigenic variations of O antigen throughout *Vibrio* forms, six immunologic groups have been established. These are arbitrarily designated as O subgroups I, II, III, IV, V, and VI. In table 8–6 the various *Vibrio* groups are depicted, which include saprophytic strains as well as *V. cholera* strains. It is important to remember that *V. cholera* strains belong to serologic group, O subgroup I, which also includes certain strains of El Tor *Vibrios*. The El Tor *Vibrios* are responsible for relatively mild diarrheal disease. The latter strains are differentiated from *V. cholera* (true cholera

Table 8–6. Vibrio Serologic Groups.

Somatic antigen	*Vibrio forms*	*Tube hemolysis (sheep cells)*
O subgroup I	*V. cholerae*	Nonhemolyic.
	El Tor vibrios	Hemolytic.
O subgroups II, III, IV, V, and VI.	Para-cholera, choleralike, and certain El Tor varieties.	Usually hemolytic.

bacilli) using the tube hemolysin test previously described. Serologic O subgroups II and VI contain miscellaneous (nonpathogenic) *Vibrio* strains as well as a few El Tor varieties. More recent studies of the O antigenic fractions of *Vibrios* by means of the so-called complete reciprocal absorption method have been made by Burrows. The results of his work reveal that the O antigenic complex consists of 13 components which are designated A, B, C, etc. The A antigen was found in all strains of O subgroup and in none of the strains of other O subgroups. For this reason, it was concluded that A antigen characterizes O subgroup I, since none of the noncholera *Vibrios* studied contained this antigenic fraction. In addition to the group specific A antigen, the true cholera *Vibrios* also were shown to contain antigen B or antigen C or both. Therefore, on the basis of the various combinations of these antigens, four immunologic types of true cholera were postulated: serotypes A, AB, AC, and ABC. Once a strain of *Vibrio cholera* has been isolated and identified by its agglutination in O subgroup I antiserum, the organism's serotype can be determined by agglutination studies employing specific antisera fractions A, B, and C. In the serologic identification of a *Vibrio* isolate suspected of being *Vibrio cholera*, a heavy normal saline suspension of 24-hour growth is first reacted with O subgroup I antiserum. This is accomplished by mixing equal droplets of suspended growth and antiserum on a glass slide (see chapter 14). If agglutination occurs, the organisms are further slide tested in monovalent antisera against the AC and AB strains. Usually, no further serological testing is necessary.

g. **Key for Identification.** Identification of *V. cholera* should be accomplished by clinical and bacteriological efforts, since the illness is seldom seen in this country. That is, when rapid dehydration, weight loss, and electrolyte loss are clinical symptoms, use TCBS agar to plate stools or exudate immediately. Serotyping should be accomplished on any isolate characteristic of the vibrio organism. Figure 8–3 shows a schematic for processing stool and exudate from suspected cholera cases.

h. **Organisms Resembling V. comma.** *V. parahemolyticus*, *V. fetus*, and *V. metchnikovii* are organisms that are similar in appearance to *V. comma*. *V. parahemolyticus* is a small halophilic, gram-negative rod, straight or slightly curved, isolated in Japan from patients suffering gastro-enteritis. It gives a negative cholera red test. *V. fetus* is a small, slender, curved rod which closely resembles the morphology of *V. comma*. It is primarily a causative agent of infectious abortion in cattle and sheep but has been isolated from various clinical conditions in man. *V. metchnikovii*, which is occasionally isolated from water sources should be mentioned in that it cannot be differentiated from *V. comma* on the basis of morphology, cultural characteristics, or biochemical tests. It causes a fatal cholera-like disease in chickens.

8–12. Bacteriology of Water. The routine bacteriologic examination of water is to determine the suitability for human consumption and general use. Water supplies should not be used until potability has been determined. Impure water may serve as an agent for the transmission of many diseases (typhoid, dysentery, gastroenteritis, and cholera). There is no routine procedure which satisfactorily detects pathogenic organisms in water. The tests for the presence of a single group of organisms (the coliform group) are used almost exclusively as an index of the degree of bacteriological pollution of water. The primary reason for testing for coliform organisms is that they are comparatively easy to isolate, whereas procedures to isolate many of the other pathogens are cumbersome and difficult. Coliform bacteria include all bacteria in the coli-aerogenes group. This group includes all aerobic and facultative anaerobic, gram-negative, nonsporeforming organisms which ferment lactose with the formation of gas and which grow aerobically on standard solid media. Coliform organisms may be of fecal or nonfecal origin. Typical of the first group is *Escherichia coli*, found in the gastro-intestinal tract of man and animals, and of the second, *Enterobacter aerogenes*, found in dust and soil. Acceptable drinking water should be substantially free of both. Qualitative tests for the coli-

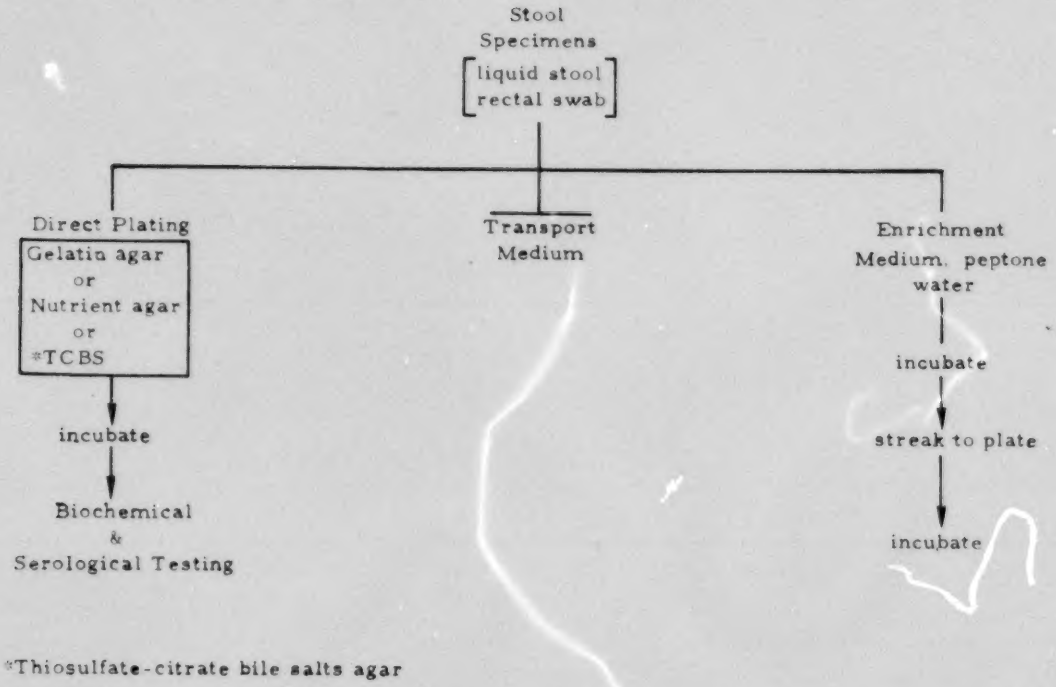


Figure 8-3. Schematic for Processing Samplings for Cholera.

aerogenes group consist of the preseumptive test, the confirmed test, and the completed test.

a. **Presumptive Test.** The presumptive test determines whether there are organisms present in the sample capable of fermenting lactose or lauryl tryptose broth with the formation of gas during 48 hours of incubation at 35°C. If gas forms, coliform organisms or other organisms capable of causing fermentation are present. If no gas forms, the sample is free of coliform organisms.

b. **Confirmed Test.** The confirmed test determines whether the organisms capable of forming gas in the presumptive test are capable of forming gas in brilliant green lactose bile broth, or of forming typical coliform colonies on endo or eosin methylene blue plates. If no gas forms in the liquid medium, the sample is free of coliform organisms.

c. **Completed Test.** The completed tests determine whether the typical or atypical coliform colonies formed on the endo or eosin methylene blue plates in the confirmed test are gram-negative and nonsporeforming, and whether they form gas when reinoculated into broth.

d. **Quantitative Estimation of Coli-Aerogenes Organisms.** Coli-aerogenes organisms can be estimated quantitatively in terms of the most probable number (MPN). The number of positive findings of coliform group organisms (either presumptive, confirmed, or completed) resulting from multiple portion decimal dilution plantings can be computed and recorded in terms of MPN. For example, assume five portions each of 10 ml, 1 ml, and 0.1 ml are used; three of the 10-ml portions are postive, one of the 1-ml portions is positive, and none of the 0.1-ml portions is positive. By the use of MPN tables in *Standard Methods for the Examination of Water and Wastewater*, American Public Health Association, Inc., 13th Ed, 1971, you can estimate the density of coliform organisms per 100 ml of water to be 11.

e. **Standard Plate Count.** The standard plate count has been included as an optional procedure because experience indicates that an approximate enumeration of the total number of bacteria multiplying at a temperature of 35°C and 20°C may yield useful information concerning the quality of the water. It may provide supporting data regarding the significance of the results of the coliform test and is useful in judging the efficiency in operation of various water treatment methods.

f. **Membrane Filter Technique.** The value of this method has been under discussion for some 19 years. Experience indicates that the mem-

brane filter technique is applicable to the examination of saline waters, but not to chlorinated waste waters.

g. **Discussion.** The results of bacteriological analyses of water must be considered in the light of all evidence at hand. In addition to the coliform index and the standard plate count, the evidence should include any information concerning the sanitary conditions surrounding the particular water source in question. Distinguishing coliforms as to fecal or nonfecal origin is not important when testing samples from closed water systems where contamination of either type would render the water unsatisfactory. However, it may be necessary to distinguish between coliform organisms as to their origin when analyzing such open water situations as bathing beaches, where soil bacteria resembling coliforms are usually present. For more detailed information concerning the bacteriology of water, refer to *Standard Methods for the Examination of Water and Wastewater*, American Public Health Association, Inc., 13th Ed, 1971.

8–13. Collection of Water Samples. Samples must be collected in sterile bottles. If the sample is chlorinated, a dechlorinating agent, such as sodium thiosulfate, must be added. If sodium thiosulfate is used, it is added to the collection bottle before sterilization in the approximate concentration of 100 mg per liter of water sample. This concentration is obtained by adding 0.1 ml of a 10 percent solution of sodium thiosulfate to each 4-ounce collecting bottle. Requirements for the storage of samples shipped to the laboratory for examination have been modified by the elimination of the need for refrigeration during transportation. Although previously required, refrigeration storage has never been properly practiced or universally used. Experience accumulated during recent years in the shipment of uniced samples by mail indicates that changes in types or numbers of bacteria during such storage are negligible. The examination of water should be started preferably within 1 hour after collection; the time elapsing between collection and examination should in no case exceed 24 hours. During the period elapsing between collection and examination, the temperature of the sample should be maintained as close as possible to that of the source of the sample at the time of sampling.

8–14. Bacteriology of Dairy Products:

a. **Categories of Disease Organisms.** Dairy products are sometimes cultured for coliform content, as are water samples. Organisms of the genera *Enterobacter* and *Escherichia*, and on occasion *Pseudomas cyanogenes*, may be found. Disease organisms which may occur in dairy products come under two categories. One group consists of those diseases of animals transmissible to man through dairy products. The other group includes those diseases which are introduced into milk from infected individuals.

b. **Sample Collection.** Samples must be collected aseptically and maintained between 1.0°C and 4.5°C until processed in the laboratory. Under proper conditions of storage, no detectable change in the bacterial flora in samples of dairy products will occur within 24 hours. Samples stored at a temperature exceeding 7°C for more than 4 hours prior to processing may give unreliable test results. For more detailed instructions concerning the techniques used for the collection and bacteriological examination of dairy products, consult the latest edition of the *Standard Methods for the Examination of Dairy Products*, published by the American Public Health Association, Incorporated.

8–15. Automation in Bacteriology. With the prevalence of computers in clinical laboratories today, it is seemingly appropriate that they be used to lighten the load in pathogen identification. If certain growth characteristics which are distinctive to certain bacteria can be programed into a computer bank and used to rapidly determine what the organism is, this would be a powerful tool for the bacteriologist.

a. **Basic Concept.** The basic concept involved here is that each bacterial organism has a definite profile place in "memory" which enables the computer to predict (through rapid elimination) the most likely organism. Each organism will be tagged with a specific code of identification. With computer speed, less time is required to determine what organism is suggested by the biochemical testing. Studies on the feasibility of automated pathogen identification have been conducted for the past several years. These studies indicate the use of computers in bacteriology shows much promise.

b. **Computer Model.** A computer model involving the calculation of "relative likelihood" as to frequency of occurrence is used to determine the most probable organism based on information input. (data matrix). The computer contains stored information in the form of "test patterns." These are compared with a

data matrix coupled with information on "expected frequency" of positive results. When a pattern of test results is presented, the likelihood that it could have been produced by an organism belonging to a diagnostic class or particular genus is estimated by calculating the probability of the observed results for each test and multiplying all probabilities together. The "likelihood" that an observed pattern of biochemical testing would be produced by an organism belonging to each of the other genera is also calculated. The program then identifies the organism as belonging to a genera or "diagnostic class" yielding the highest likelihood. A basic matrix of data is compiled using a percentage chart, and the computer assigns an identification to each pattern based on the original profile list. As with the API (Anlaytab Products, Inc) system a number of bacterial profiles have been compiled on organisms such as *Escherichia coli, Shigella spp, Citrobacter freundii, Klebsiella spp, Enterobacter spp,* and *Proteus spp.* The only drawback of automated identification systems is the possible limitation of information because of the vast number of organisms and their biochemical reactions. However, as the number of isolates in each pattern (pattern as to genus and species) increases, the quality of the "data matrix" will be further enhanced.

c. **Future Use.** Computerized identification of bacteria can be used initially to check the accuracy of identification of organisms assigned by the technologist. Also, the computer model can be very useful as a teaching tool for microbiologist and technologist. With time, the improvement of input data and data matrix will enhance the use of the computer as a means of automation in bacteria identification. This system also will be the most practical tool in taxonomy, due to speed and accuracy.

Chapter 9

SMALL GRAM-NEGATIVE BACILLI

SECTION A—HAEMOPHILUS

9-1. Introduction:

a. **Classification:**

Part 7.
Genera of Uncertain Affiliation
Genus—*Brucella*
Bordetella
Species—*pertussis*
parapertussis
bronchiseptica
Genus—*Francisella*

Part 8.
Genera of Uncertain Affiliation
Genus—*Haemophilus*
Species—*influenzae*
parainfluenzae
ducreyi
aegyptius
haemolyticus
parahaemolyticus
aphrophilus
haemoglobinophilus
citreus
Genus—*Pasteurella*

b. **Morphology.** The genus *Haemophilus* consists of a group of very small, gram-negative, bacilli which are non-sporeforming, nonmotile, and hemophilic (blood-loving). Morphologically these forms range from very short, slender, coccobacillary forms to longer, threadlike filaments. Although in many instances the various strains of *Haemophilus* spp are microscopically indistinguishable, the better known forms have been shown to exhibit some cellular features of differential value.

(1) *Haemophilus Influenzae (Pfeiffer's Bacillus)*. This species is usually observed as very small bacilli, rarely exceeding 1.5 microns in length and 0.3 microns in breadth. The cells predominantly occur as coccobacillary forms, although longer slender bacilli may be observed. There is a marked tendency for the organism to produce long thin filaments, or other aberrant forms, in culture. The organisms are more difficult to stain than most bacteria. In smears of young mucoid (M) colonies, definite capsules are exhibited. These capsules are rapidly dissolved by autolytic enzymes as the culture becomes older.

(2) *Haemophilus hemolyticus*. Strains of this species vary from small rods resembling *H. influenzae*, to larger bacillary forms occurring as irregularly shaped filaments. In addition, many pleomorphic forms are often observed which may be spheroid, tenpin-or dumbbell-shaped, or even clubshaped in appearance.

(3) *Haemophilus ducreyi*. When observed in Gram-stained smears of tissue exudates, Ducrey's bacillus will appear as short ovoid rods in end-to-end pairs (diplobacilli) or short chains. Individual cells are about 1 to 1.5 microns in length and 0.6 microns in breadth.

(4) *Other Haemophilus Species. H. parainfluenzae, H. parahaemolyticus*, and *H. haemoglobinophilus* are microscopically similar to *H. influenzae*. These organisms, when isolated from clinical specimens, are differentiated from other *Haemophilus* spp on the basis of cultural and biochemical characteristics described in the sections which follow.

(5) *Bordetella*. The members of this genus are most often seen as small coccoidlike bacilli, occurring singly, in pairs, and sometimes in short chains. Morphologically, they are similar to the hemophilic bacteria. Previously the species included in this genera were placed in the genus *Haemophilus* as *H. pertussis, H. parapertussis*, and *H. bronchisepticus*, based on this morphological similarity.

9-2. Pathogenicity:

a. **Haemophilus influenzae.** The encapsulated strain of *H. influenzae* may cause pharyngitis, otitis, sinusitis, pneumonitis, or meningitis. Meningitis is extremely rare, occurring primarily in children under 3 years of age. The nonencapsulated variety of *H. influenzae* is considered to be normal flora in the upper respiratory tract of adults.

b. **Haemophilus ducreyi.** This Hemophilus species is the etiologic agent of a venereal disease characterized by the formation of ragged, soft ulcers in the genital region. The regional lymph nodes of the groin may become secondarily infected and are referred to as buboes. The ulcers differ from typical hard chancre of primary syphillis lesions in that their edges are soft. Such localized infections are called soft chancres or chancroids. The buboes may rupture and form secondary ulcers. Chancroid ulcers often become infected with other bacteria (*H. haemoglobinophilus* and *H. citreus*).

c. **Bordetella.** The pertussis bacillus is responsible for infections of the tracheal and

bronchial epithelium; the disease is known as whooping cough. As a result of multiplication in these areas, the organisms interfere with the ciliary action of endothelial cells and cause episodes of uncontrollable coughing accompanied by inspiratory whoops. Whooping cough is largely confined to children and is highly contagious. *B. parapertussis* and *B. bronchiseptica* are responsible for a mild, often subclinical form of whooping cough.

d. **Infection From Other Haemophilus Species.** Although the aforementioned *Haemophilus* spp are more important from a clinical standpoint, the significance of other species with respect to human infection must also be considered. These organisms are as follows:

(1) *H. parainfluenzae* is a normal inhabitant of the human respiratory tract and is encountered as a disease agent only in bacterial endocarditis.

(2) *H. haemolyticus* is present in the normal respiratory tract; occasionally it is associated with nasopharyngeal infections of children.

(3) *H. aegyptius* is the etiologic agent of a highly contagious conjunctivitis (pink eye), especially in children.

9–3. Growth Requirements:

a. **Haemophilus Species:**

(1) *Temperature and Gas Requirements. Haemophilus* spp grow best at 37°C in a candle jar.

(2) *Blood Factors.* The group is composed of hemophilic or hemoglobinophilic species. *Haemophilus influenzae* and closely related species require two factors in blood for growth.

(a) *X Factor.* One factor, hemin (designated as X), is a heat-stable derivative of hemoglobin.

(b) *V factor.* The other factor, nicotinamide adenine cleotide (designated as V), is a heat-labile fraction of blood consisting of coenzyme I or II. V-factor may be replaced by NAD or NADP. The V-factor also is formed by yeasts, staphylococci, and certain other microorganisms.

(3) *Isolation Media Requirements.* Blood should be incorporated in isolation media for the *Haemophilus* spp requiring X and/or V factors.

(a) Some variation exists in the ability of certain *Haemophilus* spp to grow on human blood. Practically speaking, most human blood is satisfactory for use in isolation media.

(b) Rabbit, guinea pig, or sheep blood may be substituted for human blood. On rabbit or guinea pig blood, hemolytic species of Haemophilus (*H. haemolyticus* and *H. parahaemolyticus*) may be confused with beta hemolytic streptococci without the aid of gram-staining. Sheep blood contains substances which inhibit the growth of *Haemophilus* spp and can be used only for the preparation of chocolate agar.

(4) *Preparation of Chocolate Agar:*

(a) The inhibitory substances in sheep blood are destroyed by the necessary temperature (70°C to 75°C) required of the sterilized agar base for lysis of red blood cells, in making chocolate agar.

(b) Since heat-labile V factor also is destroyed in preparing chocolate agar, it must be restored to the medium. This is accomplished by adding enrichment supplements A or B to chocolate agar at a temperature of 40°C.

(c) If enrichment supplements are not available, heavily inoculate a plain chocolate agar plate with the specimen presumed to contain a Haemophilus spp.

(d) Immediately thereafter, apply one or two streaks of staphylococcal organisms at right angles to the primary streaks. After 24 to 48 hours of incuation, *Haemophilus influenzae* or other species requiring V factor will appear as small colonies growing in close proximity to the colonies of staphylococci. This is referred to as "satellitism" and results from V factors being formed by the *Staphylococcus* spp. Diffusion of V factor into the surrounding medium provides a readily available enrichment source for the *Haemophilus* sp, and little or no growth of the organism will occur except along the staphylococcal growth line.

(5) *Bacitracin Disk.* Recently introduced techniques prescribe the placing of a paper disk saturated with bacitracin, saponin, and a yeast solution rich in V factor on a sheep-blood agar plate. The bacitracin acts to inhibit all gram-positive and some gram-negative contaminants. The saponin diffuses from the disk into the medium and disrupts the blood cells, releasing X factor. Excess V factor diffuses from the disk into the medium and is also available to the growing hemophiles. Growth, using the above technique, is better if rabbit or guinea pig blood agar is used.

(6) *Cultural Characteristics:*

(a) *H. influenzae.* H. influenzae strains isolated from most pathologic sources occur in the so-called S or smooth phase. The S colonies are raised, slimy, mucoid, confluent, and nonhemolytic. Microscopic examination will reveal the presence of capsules. The R or rough

colonies are small, nonhemolytic, transparent to translucent, and present a discrete dew-drop appearance. The cells of the R phase are nonencapsulated. The S phase colonies are readily differentiated from R phase colonies in that S colonies are larger (2 μm to 4 μm) and more slimy and irregular than the well-defined colonies of the R phase. After two or three transfers on culture media, the encapsulated S colonies revert to the nonencapsulated R phase.

(b) *H. haemolyticus*. The colonies of *H. haemolyticus* may be confused with the colonies of beta streptococci because of similarity of gross morphology and hemolysis. All colonies appearing to be beta streptococci must be verified by gram-staining.

b. **Bordetella Species.** *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* do not require X or V factors for growth. For this reason, these species have been removed from the genus *Haemophilus*. Nevertheless, blood is definitely stimulatory to members of the *pertussis* group and should be incorporated in isolation media. The glycerin-potato-blood agar of Bordet and Gengou is most often used for cultivation of these organisms. The growth of *B. pertussis* on this medium is slow, usually requiring from 3 to 5 days' incubation. The colonies are small and dome-shaped, possessing a gray metallic luster, resembling mercury droplets. Although the colonies of pertussis bacilli are beta haemolytic, the zone of haemolysis is difficult to observe since Bordet-Gengou agar contains 15 to 20 percent blood. In table 9–1, the specific requirements with respect to X and V factors are given, along with the hemolytic activity for the more common *Haemophilus* spp.

9–4. Isolation Procedures. The following procedures are satisfactory for isolating all species of *Haemophilus* except *H. ducreyi*:

a. **Rapid Screening:**

(1) Specimens from areas contaminated with mixed flora, such as the nasopharynx and bronchus, are best cultured using a selective screening technique.

(2) Heavily inoculate such specimens on a sheep-blood agar plate.

(3) Place a paper disk containing bacitracin, saponin, and yeast extract on the plate.

(a) Gram-positive flora are inhibited by the bacitracin; saponin stromatolyzes the blood cells, releasing their hematin and NAD; and the added yeast extract insures abundant growth.

(b) Disks used in the procedure can be prepared by soaking bacitracin disks (2 units/disk) in a solution containing 3 percent saponin and 5 percent dried yeast extract. Dry the prepared disks at 37°C and store for up to 4 months at 4°C.

(c) Disks are also available commercially (Difco).

(4) Incubate at 37°C in 10 percent CO_2.

(5) Hemophilic bacteria, if present, are found in the transparent zone surrounding the disk.

b. **Blood Cultures.** *Haemophilus influenzae* and other species are present in the blood in meningitis, endocarditis, and obstructive laryngitis. Other species are found mostly in endocarditis and rarely in meningitis. Isolation can be made using standard blood culture bottles containing CO_2. Some reports indicate that the addition of a sterile solution of a stromalyzing agent (for example, saponin) to the blood improves growth.

c. **CSF and Other Fluids.** Cerebrospinal, synovial, and pleural fluids, as well as other relatively uncontaminated specimens, should be heavily inoculated on sheep blood agar and separate disks containing saponin and yeast extract applied 3 to 4 mm apart. This procedure allows presumptive identification of the *Haemophilus* species present. Species requiring only X factor will grow abundantly around the saponin disk. Those requiring only V factor show good growth around the yeast extract disk. Those requiring both factors grow well in the space between the disks. The disks (available commercially) used in this procedure are prepared by soaking plain paper disks separately in solutions of saponin and yeast extract, as given in the rapid screening procedure.

d. **Eye and Ear Specimens.** Specimens taken in acute conjunctivitis and those of otitis are cultured using either of the procedures above. If a gram-stained smear shows little mixed flora is present, the method recommended for fluids should be used. If much mixed flora is present, use the screening procedure. Organisms to be expected are *H. aegyphius* from eye discharges and *H. aphrophilus* and *H. influenzae* from ear cultures.

9–5. Differentiation Procedures:

a. **Stained Smears.** Gram-stained smears of spinal fluid, pleural fluid, synovial fluid, and eye and ear discharges should be made and examined immediately. If short gram-negative rods or coccobacillary forms are seen, *H. influenzae* can be tested for immediately, using type-specific serum in a *quellung* (capsular swelling)

Table 9–1. Growth Requirements and Hemolytic Activity of *Haemophilus* spp.

Species	Specimens	Isolation media	Growth characteristics	X Factor	V Factor	Hemolysis
Haemophilus influenzae-------	Blood ----------	Trypticase soy and thioglycollate broths.	Subcultured to rabbit blood agar or supplemented chocolate agar; M phase colonies; mucoid, raised, confluent, transparent to translucent.	+	+	-
Haemophilus influenzae-------	Spinal fluid sediment, sputum, or swabs from throat, nasopharynx or sinus passages	Rabbit blood agar or supplemented chocolate agar.	M phase colonies as described above, or S phase colonies; small, colorless, transparent to translucent and nonhemolytic (dewdrop colonies).	+	+	-
Haemophilus aegyptius --------	Eye swabs ----------	Rabbit blood agar or supplemented chocolate agar.	Indistinguishable from S phase colonies of *H. influenzae*.	+	+	-
Haemophilus ducreyi ----------	Swab materials from chancroid or aspirants from buboes.	Fresh, sterile, clotted rabbit blood.	Chains of small, Gram-negative bacilli in palisades or "schools of fish" arrangement (no growth on agar media).	+	-	+/-
Haemophilus hemolyticus ------	Throat culture Broncial secretion.	Chocolate agar. Blood agar (fresh).	Bacillary forms with tangled filaments.	+	+	+
Haemophilus parainfluenzae ---	Bronchial specimens ---	Chocolate. Blood agar (fresh).	Rods with sometimes pointed ends.	-	+	+ or -
Haemophilus parahemolyticus --	Bronchial nasopharynx -	Chocolate agar. Blood agar.	Stout rods or tangled filaments.	-	+	+
Haemophilus hemoglobinophilus	No specificity	Chocolate. Blood agar.	Small to long rods and filaments.	+	-	-

type reaction. If the test is negative or to confirm a positive test, perform a *X-V factor* determination as below.

b. **Iridescence.** Fildes' or Levinthol's agar can be used to inspect for *H. influenzae's* ability to radiate a shiny bluish-tinged color in reflected (oblique) light. In normal light these colonies will appear white/grayish in color. This property is characteristic of virulent type b, *H. influenzae*. It is also produced by growth of pathogenic *H. parainfluenzae*.

c. **Growth Factor Determination.** Organisms presumptively identified as hemophilic bacteria are tested to confirm their growth factor requirements. This is accomplished by subculturing a pure inoculum to a casein soy digest, meat infusion, or Casman agar plate and placing separate paper disks or strips containing hemin and NAD 3 to 4 mm apart. The disks and strips are available commercially (Difco; BBL) or they can be prepared by soaking blank paper disks with solutions containing 500 μg per ml hemin and 100 μg per ml yeast extract, respectively. Growth in the near vicinity of a disk indicates that factor is required. Growth between the spaced disks indicates both factors are required. Alternately, the test can be performed by streaking one-half of a sheep-blood agar plate with *S. aureus* and both sides with the inoculum. Growth on the side with the *S. aureva* streak indicates X and V factors are required. Growth on the unstreaked side indicates only an X factor requirement.

d. **Red Cell Agglutination Test.** This test aids in separating *H. influenzae* from *H. aegyptius* because growth factor requirements are identical for these species. *H. aegyptius* stronly agglutinates a suspension of human cells while *H. influenzae* produces weak agglutination is best described with the use of a small concave microscope mirror. The mirror is held under the tube and viewed from a 45° angle above, so that the image of the cells is enlarged.

e. **Capsule Stain.** Most pathogenic strains of *H. influenzae* are capsulated. Nonvirulent strains and *H. aegyptius* are noncapsulated. Stains must be made on young cultures because of the high rate of autolysis exhibited by the hemophilic bacteria, particularly *H. influenzae*. Loffler's methylene blue or 10 percent aqueous basic fuchsin gives satisfactory results. Capsule stains must be accompanied by a gram-stained smear because of the similarity to *Streptococcus pneumoniae*.

f. **Nitrate and Catalase.** With the exception of *H. ducreyi*, all species of the hemophilic bacteria reduce nitrate to nitrite, and all are catalase-positive.

g. **Quellung Reaction.** This capsular swelling test is performed on pure cultures of *H. influenzae* and on specimens of body fluids that show relatively little contamination with other flora:

(1) Make a slightly turbid saline suspension of a colony, or directly deposit a small drop of fluid specimen on a slide.

(2) Add a drop of antiserum and then a drop of Löffler's methylene blue.

(3) Cover with a petrolatum-rimmed coverslip, seal, and let stand for 15 minutes.

(4) Examine under a microscope in diminished light.

(5) Use polyvalent antiserum initially, and if positive follow with strain-specific antisera.

(6) Alternately, a precipitin test can be performed; however, a positive quellung reaction is usually considered diagnostic.

h. **Carbohydrate Tests.** Results of carbohydrate tests are too variable to be of much use. The above-described procedures are satisfactory for establishing identification.

9–6. Other Haemophilus Species:

a. **Haemophilus parainfluenzae.** This species differs from *H. influenzae* in that only V factor is required for growth. The organism is frequently found in the normal throat and is usually noncapsulated. A few strains isolated in disease are capsulated and show iridescence in reflected light. This species is usually nonpathogenic but can cause pharyngitis, bacterial endocarditis and occasionally bronchitis. The species is the second most frequently isolated hemophilic species.

b. **Haemophilus haemolyticus.** This species requires both X and V factors; it is inhibited by sheep blood. It produces β-hemolysis on rabbit or guinea pig blood. It rarely causes disease but has been isolated in bacterial endocarditis.

c. **Haemophilus parahemolyticus.** The organism requires only V factor and also is hemolytic on rabbit or guinea pig blood. It has been isolated in bacterial endocarditis.

d. **Haemophilus aegyptius.** The organism causes conjunctivitis, especially in warm climates. It requires both X and V factors and is easily confused with both *H. influenzae* and *Moraxella lacunata*. It differs from the former in that human red blood cells are strongly agglutinated and from the latter in that *M.*

lacunata grows on Löffler's medium and produces characteristic pits or lacunae.

9–7. Haemophilus ducreyi. This organism is difficult to isolate because of its unusually strict nutritional requirements. It will grow on Bordet medium prepared with sheep and human blood. It causes the veneral disease known as chancroid or soft chancre. The disease is presumptively diagnosed by finding characteristic small, gram-negative ovoid rods in pairs or strands on a smear made from a lesion. It can be cultured, however, by inoculating a tube containing 1.2 ml of freshly clotted rabbit blood, or the patients's own blood, and incubating it at 37°C in increased CO_2 for a minimum of seven days. Smears of this culture typically show small, gram-negative rods arranged in strands resembling "schools of fish." Subcultures can be made on chocolate agar, also suitable for maintaining stock cultures. The organism requires X factor only. Identification can be confined with type-specific antiserum.

SECTION B—BORDETELLA

9–8. Pathogenicity:

a. Etiology of pertussis. *Bordetella* organisms cause whooping cough (pertussis), a disease of childhood. The disease is not often seen in adults because 9 out of 10 children acquire some form of the disease and as a result develop long-lasting immunity to reinfection, and the usage of DPT immunization. Also, effective immunization is available to prevent the most serious form of the disease, namely that caused by *B. pertussis*. The other members of the genus, *B. parapertussis* and *B. bronchiseptica*, cause milder, less serious forms of the disease.

b. **Course of the Disease.** Whooping cough usually starts out as sneezing or a mild cough and progresses in 10 to 14 days to lower respiratory involvement, producing the characteristic "whooping" type of cough. The patient in serious cases develops bronchopneumonia and in extreme cases, encephalitis. Death as a result of the disease occurs most often in very small infants and is due mainly to thick mucus, which plugs the bronchi, causing anoxia and convulsions.

c. **Immunity.** Acquired immunity gives permanent protection. Immunization against *B. pertussis*, the severest form of the disease, is recommended for children at age 3 months, with boosters at 1, 3, and 5 years. Immunization against *B. parapertussis* and *B. bronchiseptica* is not given because these organisms cause a mild form of the disease that is usually not fatal.

9–9. Growth Requirements:

a. **Growth Factors.** *Bordetella* organisms do not require X and V factors for initial isolation. *B. parapertussis* and *B. bronchiseptica* can be subcultured on media that do not contain X or V factors.

b. **Bordetella pertussis.** This is an extremely fastidious organism and will not grow on media containing peptone unelss blood or charcoal is present. These substances probably act to inactivate inhibitory substances (fatty acids) in peptone-based media. Selective cultivation of *B. pertussis* is accomplished by growing the organism on modified Bordet-Gengou media (BBL), which contains no peptone, glycerol, potato extract, and up to 50 percent defibrinated blood. Penicillin is added directly to this medium to inhibit mixed flora. *B. bronchiseptica* grows better on blood agar than does *B. parapertussis*.

c. **B. bronchiseptica.** This species will grow on MacConkey's and Salmonella-Shigella (SS) agar.

9–10. Isolation and Identification Procedures:

a. **Specimen Collection.** Specimens should be collected early in the first stages of the disease. After 4 to 5 weeks, specimens may yield negative cultures.

(1) *Cough Plate.* This is the traditional method of collecting whooping cough specimens. The patient is permitted to cough directly on the moist surface of a plate of Bordet-Gengou medium. Spray and droplets from several deep coughs are deposited on the agar.

(2) *West Swab.* This technique is the recommended method of collecting whooping cough specimens.

(a) Insert a West swab prepared from a piece of thin, flexible wire into a nostril, and pass it through to the nasopharynx.

(b) Pass the material collected on the swab several times through a drop of penicillin solution (1000 units/ml), placed on the surface of a Bordet-Gengou agar plate.

(c) Smear the inoculated antibiotic droplet over the surface of the plate with a platinum loop.

(d) If difficulty is encountered getting the swab through the nostril, use the cough plate technique.

b. **Selective Isolation of B. pertussis.** *B. pertussis* can be selectively isolated from mixed flora by using modified Bordet-Gengou medium. This medium is similar to the original medium except that peptone is left out of its formulation.

(1) Enrich the medium with 20 to 50 percent fresh sheep, rabbit, or human blood.

(2) Add penicillin directly to the agar (0.25 to 0.5 units/ml) to inhibit the mixed flora, such as spreaders, which otherwise quickly overgrow the pathogen.

(3) Incubate in a moist atmosphere at 36°C ± 1° for up to 6 days. Growth normally does not occur before 48 hours, but usually occurs before 72 hours.

(4) Cut out and remove with a sterile loop or scalpel spreaders and contaminants, which appear quickly because of the high blood concentration; reincubate the plate.

(5) Modified Bordet-Gengou is available commercially.

c. **Routine Isolation Procedure.** The following technique is recommended for culturing any of the three *Bordetella* species:

(1) Isolate initially on Bordet-Gengou medium.

(2) Inspect growth, with hand lens if necessary, and make gram stain. *B. pertussis* and *B. bronchiseptica* are small (1.0 by 0.3 to 0.5 μm) coccoid-shaped rods found singly and in pairs. *B. parapertussis* is similar, but sometimes the rods are larger.

(3) Colonies of *Bordetella* are small, convex, shiny, and entire; they resemble a mercury droplet or pearl surrounded by a zone of hemolysis. Hemolysis may require 4 to 5 days before appearing. Colonies of *B. parapertussis are* usually slightly larger than the other two species.

(4) If after 72 hours, growth is present but too scanty to be used in differentiation tests, subculture to a fresh plate of Bordet-Gengou agar. Subculture growth is usually more abundant.

d. **Differentiation:**

(1) The three species of *Bordetella* can be differentiated on the basis of:

(a) Growth in plain media containing peptone but no blood.

(b) Motility.

(c) Citrate utilization.

(d) Brown pigment production on peptone media.

(e) Nitrate utilization.

(f) Indole production.

(2) Results of biochemical tests are shown in table 9-2.

e. **Serological Confirmation.** Identification of *B. pertussis* can be confirmed by a slide agglutination test using specific antiserum.

(1) Make a turbid, even suspension of test organisms from the growth on the Bordet-Gengou plate in a drop of saline on one end of a slide.

(2) Mix several loopfuls of the suspension with several loopfuls of diluted antiserum.

(3) *B. pertussis* causes immediate agglutination.

(4) Run a negative saline control (minus antiserum). This preparation should show no clumping.

SECTION C—BRUCELLACEAE

9-11. Introduction. Members of the genera *Brucella, Pasteurella,* and *Haemophilus-Bordetella* are the principal representatives of the small gram-negative rods. The organisms of these groups are minute, ovoid bacilli, which are nonmotile (except *B. bronchiseptica*) and nonsporeforming. These organisms have complex nutritive requirements which necessitate the use of enriched media for cultivation, again with the exception of *B. bronchiseptica.*

9-12. Classification and Morphology:

a. **Classification:**

Part 7. Gram-Negative Aerobic Rods and Cocci

Table 9-2. Differential Characteristics of *Bordetella* sp.

Organisms (species)	Growth on blood-free peptone agar	Browning of peptone agar	Citrate	Produces Urease	Reduces Nitrate	Indole	Motility
B. pertussis	-	-	-	-	-	-	-
B. parapertussis	-	+	+	+	-	-	-
B. bronchiseptica	+	-	+	(within 4 hrs)	+	-	+

(All) Reactions: - negative
+ positive

Genera of Uncertain Affiliation
Genus—*Brucellae*
Species—*abortus*
—*suis*
—*melitensis*

b. **Morphology.** These organisms are predominantly small coccobacillary forms ranging from 0.4 to 3.0 μm in length and 0.4 to 0.8 μm in breadth. Fresh isolates from disease are encapsulated and form smooth mucoid colonies on agar media. The cells occur singly, in pairs, or in short chains. *Brucella spp* do not possess flagella, nor do they form spores.

9–13. Cultural Characteristics. On trypticase soy agar, colonies of *Brucella spp* are small, translucent, smooth, glistening blue-gray in color, and possess entire margins.

9–14. Growth Requirements. *Brucellae* require complex media for growth. Although many special media have been devised and recommended for cultivating *Brucella spp*, trypticase soy broth and agar are used with considerable success. *Brucella abortus* can be cultivated only from clinical specimens in an atmosphere of approximately 10 percent carbon dioxide. *Brucella suis* and *Brucella melitensis* will grow with or without increased carbon dioxide. Prolonged incubation (several weeks) at 37°C is often necessary for initial isolation of these organisms. However, on subculture, growth usually occurs within 3 or 4 days.

9–15. Pathogenicity. The brucellae cause brucellosis, a disease primarily of domesticated animals, which is secondarily transmissible to man (undulant or Malta fever). *B. abortus*, *B. melitensis*, and *B. susis* are all pathogenic to man. Following ingestion of raw milk from infected animals, the organism may invade the oral mucosa or the lining of the intestinal tract. Infections also occur as a result of direct contact with the organism or infected tissues. Cases of pulmonary brucellosis suggest that infection may result from inhalation of the organism.

a. The highest incidence of brucellosis is found among persons, such as veterinarians or butchers, whose occupations call for dealing with animals.

b. After entry into the human host, the organisms progress by way of the lymphatic channels to the thoracic duct. They enter the bloodstream and are widely disseminated to various tissues including the liver, spleen, bose marrow, and throughout the reticuloendothelial system. The organisms form multiple intracellular abscesses in the particular tissue involved. Osteomyelitis or meningitis may occur.

c. Characteristic symptoms begin about 1 to 6 weeks following infection, subsiding within 3 months and remaining dormant in deep tissues as long as the individual's resistance is high. At the height of infection (after 6 weeks) acute febrile episodes occur as a result of the organism's periodicity in the bloodstream. This is a good time for serological confirmation of brucellosis.

9–16. Identification:

a. The specimess usually examined for *Brucella spp* are blood samples taken during the febrile phase of the disease. Lymph aspirations, biopsy materials, spinal fluid, or swab specimens of deep lesions and bone marrow also may be examined.

b. The procedures for collection and cultivation have been previously described (see chapter 2). CAUTION: BRUCELLAE ARE VERY PATHOGENIC, and strict asepsis must be practiced at all times; use a hood when handling such samples.

c. Specimens other than blood should be inoculated to trypticase soy agar and broth. All specimens must be cultivated at 37°C under 10 percent carbon dioxide.

d. Although the gram-negative, coccobacillary cells of *Brucella spp* may be observed in sputum or deep tissue fluids, such smears are rarely of diagnostic value since other bacteria may exhibit similar morphology.

e. The blood culture bottle (BBL, Castaneda, etc) should be examined for small, bone-gray colonies of *Brucella spp* which may be present on the trypticase soy agar slant. Gram stains of the colonies should reveal short gram-negative rods under microscopic examination.

9–17. Confirmation of Brucella. The following studies are performed on cultures presumed to be *Brucella spp:*

a. Inoculate two trypticase soy agar slants and incubate one tube in a candle jar. Place the second slant at room temperature. *B. abortus* requires 5 percent carbon dioxide for growth, while *B. melitensis* and *B. suis* will grow without carbon dioxide in the environment.

b. Make stab inoculations of glucose, lactose, and sucrose O/F basal media. *Brucella spp* weakly ferment glucose and do not ferment lactose or sucrose. *Salmonella spp* and other gram-negative rods will strongly ferment at least one of these sugars.

c. Streak two trypticase soy agar slants (one containing 1:200,000 dilutions of thionine, the other 1:100,000 dilutions of basic fuchsin) to check for inhibition of growth following incubation.

d. Perform motility test using trypticase semisolid media. *Brucella spp* are nonmotile while other gram-negative bacilli encountered in blood are usually motile.

e. Heavily inoculate suspected *Brucella spp* to Christensen urea agar. *Brucella spp* are urease positive.

f. Inoculate a tube of trypticase soy broth and suspend a strip of sterile lead acetate paper over the medium. Viable *Brucella spp* will produce hydrogen sulfide for variable periods of time. Hydrogen sulfide production is evidenced by the lead acetate paper's turning black. Change the paper daily during several days of incubation, and record hydrogen sulfide production for the organism concerned. A summary of the previously mentioned procedures is provided in table 9–3.

SECTION D—PASTEURELLA (*Francisella-yersinia*)

9–18. Classification and Morphology:

a. **Taxonomy:**

Part 7. Gram-Negative Aerobic Rods asd Cocci

Genera of Uncertain Affiliation
Genus—*Francisella*
Species—*tularensis*
novicida

Part 7. Gram-Negative Facultatively Aerobic Rods

Genera of Uncertain Affiliation
Genus—*Pasteurella*
Species—*multocida* (septica; *cholerae-gallinarum*)
haemolytica
ureae

Part 8. Family I Enterobacteriaceae
Genus XI—*Yersinia*
Species—*pestis*
enterocolytica
pseudotuberculosis

b. **Morphology.** *Pasteurella* are small ellipsoidal to elongated, bipolar-staining, gram-negative, motile rods. Two nonmotile species, *P. tularensis* and *P. novicida*, have been reclassified under the genus *Francisella*, named after Dr. Edward Francis, who discovered the organism in Tulare County, California. These species are now called *F. tularensis* and *F. novicida*. *Yersinia pestis*, formerly *Pasteurella pestis*, has been placed in a separate genus (*Yersinia*, within the family Enterobacteriaceae) for the plague bacillus, along with the related organism *P. pseudotuberculosis*. *Y. pestis* is a short, plump, ovoid rod ranging from 0.3 to 1.25 μm in length. The organism is encapsulated and characteristically exhibits bipolar staining. Considerable pleomorphism is exhibited in older cultures; they vary from coccoid shapes to larger, elongated bacilli, which are often swollen. *Y. pseudotuberculosis* resembles the plague bacillus morphologically, but is differentiated by its motility at 18°C to 22°C when stabbed in soft agar. *Francisella tularensis* is usually much smaller, 0.3 to 0.7 μm in length, and 0.2 μm in breadth. In young cultures the coccoid form predominates while in older culture the bacillary form is more prevalent. The latter cells are usually pleomorphic asd may be bipolarly stained. When observed in exudates, the organism is encapsulated.

9–19. Cultural Characteristics:

a. **Yersinia:**

(1) *Y. pestis* will grow on trypticase soy blood agar after 48 hours incubation. Colonies will appear as small (0.1 to 0.2 μm), round, glistening, transparent, colorless colonies, which are droplet-like and nonhemolytic. Older colonies become larger, more opaque, and develop grayish-yellow centers with gray-white edges. Broth cultures appear turbid or may contain flocculent growth.

(2) *Y. pseudotuberculosis* will grow on blood agar and MacConkey's agar. Colonies may appear in 24 hours as smooth, grayish-yellow, translucent growths; upon continued incubation, raised colonies with apaque centers and light, serrated margins may appear.

(3) *Y. enterocolytica* produces tiny, translucent, homogeneous colonies with a hazy rim on SS agar after 18 hours incubation. Growth is very slow, requiring at least 48 hours incubation at 35°C to 37°C on MacConkey, EMB, or desoxycholate agar.

b. **Francisella tularensis.** Primary growth of *F. tularensis* requires cysteine in the medium, as for example, cysteine glucose agar or cysteine heart (extract) agar. Thiamine heart agar is also satisfactory. Isolation of the organism is enhanced by the addition of antibiotics to retard growth of "normal flora" in specimens cultured for *F. tularensis*. Penicillin, polymyxin B, and cyclohexamide are incorporated as the antibiotic additive used as the inhibitor in the media. *F. tularensis* requires from 4 to 7 days before growth is observed. In young cul-

Table 9–3. Identification of *Brucella* spp (Differentiation).

Species	*Carbon Dioxide Requirements*	*Carbohydrate fermentation*			*Growth in presence of*		*Motility*	*Urease*	*Hydrogen Sulfide*
		Glucose	*Lactose*	*Sucrose*	*Basic fuchsin*	*Thionine*			
Brucella abortus	Required for growth (usually).	+ −	−	−	+	−	−	+	+ for 2 days
Brucella suis	Not required for growth	+ −	−	−	−	+	−	+	+ for 4 days
Brucella melitensis	Not required for growth	+ −	−	−	+	+	−	+	+ or − for 1 day

NOTE: 1. All cultures are incubated in a candle jar for 4 days except cultures for carbon dioxide differentiation.
2. When fermentation media are incubated in an atmosphere of increased carbon dioxide, an acid reaction results, regardless of whether or not the carbohydrate is fermented. Therefore, following incubation, such cultures must be allowed to stand at room temperature overnight before reading. This will allow dissipation of the absorbed carbon dioxide and subsequent reversion of the indicator to only that reaction produced by the organism.

tures the colonies are very tiny although gram-stained smears of fluid from the base of the slant reveal numerous cells. Later, relatively heavy growth of small, gray, transparent to translucent, mucoid colonies develops. Subcultures to cysteine (glucose) blood agar yield abundant growth within 2 to 3 days. The colony appearance of *F. novicida, the* "new killer," is similar to that of *F. tularensis.*

c. **Pasteurella grows** in any of the three colony forms—smooth, mucoid, and rough. Colonies are 1.0 to 1.5 μm in diameter and often are iridescent in oblique light. Cultures have a distinctive odor; in the case of *P. multocida,* they smell like burning hair. Hemolysis may be absent or complete (*B*eta). Biochemical characteristics of the *Pasteurella* species are listed in table 9–4.

9–20. Growth Requirements. *Pasteurella* spp will grow on conventional nutrient agars while *Yersinia* spp are not fastidious with respect to nutritive requirements, although trypticase soy agar and broth are recommended for its primary cultivation. Once isolated, the organism may be subcultured on ordinary media. *Francisella* spp will not grow on ordinary media and also may fail to grow on enriched blood agar unless the amino acid cystise or cysteine is added. Cultivation is most successful when using cysteine-glucose blood agar slants. Both *Yersinia* spp and *Francisella* spp seem to have an optimum temperature of 37°C in reduced oxygen tension.

9–21. Pathogenicity:

a. **Yersinia pestis** is the etiological agent of plague, a disease primarily of rodents, which is secondarily transmitted to man. The bacilli enter the body and spread via the lymphatic system, causing the nodes to become inflamed and hemorrhagic. The enlarged lymph nodes form buboes, which are characteristically located in the groin or axilla. The organism, using the blood as its vehicle, also may spread to other tissues, including the spleen, liver, and lungs. Death can result from meningitis or septicemia. Persons with lung involvement can spread the organism to others by coughing and sneezing. Primary pulmonary infections (pneumonic plague) are fatal if untreated. *Y. pseudo*-tuberculosis has been isolated in septicemia, cases of mesenteric lymphadenitis, and some cases of esteritis. *Y. enterocolytica* infections are involved in cases of bloody diarrhea in young children, septicemia, meningitis, indwelling catheter site infections, cholecystitis, and various abcesses.

b. **Francisella tularensis** is the causative agent of tularemia, primarily a disease of rodents. Rodents include rabbits, beavers, ground squirrels, muskrats and other animals. The reservoir of infective agents is maintained in rodents by infected insect bites from animal to animal. Man may contract tularemia directly by handling infected animal flesh or indirectly from insect bites. The main source of infection for man is the rabbit. The course of the disease is similar to that of plague with the addition of eye involvement, such as oculoglandular infections and conjunctival compliations. In some cases, there are no signs of localized involvement but only a clinical picture of febrile systemic illness.

c. **Yersinia pseudotuberculosis** (measuring 0.8 to 2.0 μm) causes a tubercular-like infection in a wide variety of animals. It is prevalent in soil, water, milk, and animal fodder.

d. **Pasteurella multocida** (measuring 0.15 to 0.25 μm) causes a hemorrhagic septicemia in warm-blooded animals, which harbor the organism in the mucous membrance during the course of infection. Persons infected by bites from such animals present clinical symptoms of meningitis, enteritis, conjunctivitis, and sinusitis.

9–22. Identification:

a. **Specimen Culture:**

(1) *Plaque* (*Y. pestis*). When plague suspected, materials aspirated from buboes and sputum should be examined. Blood specimens

Table 9–4. Identification and Differentiation of *Pasteurella* spp.

	Characteristics/Reactions					
Species	Hemolysis	Catalase	Oxidase	Urease	Growth on MacConkey	Indole
P. multocida	-	+	+	V	-	+
P. [illegible]	α	+	+	++	-	-
P. haemolytica	β	+	+	-	+	-

also should be cultured early and late in the course of the disease during temperature spikes. At autopsy, material from lesions and inflamed areas of internal organs, especially the spleen, will yield the organism.

(2) *Tularemia* (*F. tularensis*). Cultures should be made from lymph fluid, blood, sputum, and materials from localized lesions. Blood cultures may be taken repeatedly during the first week of infection. Multiple sampling is suggested during early stages to enhance chances of recovery of the organism. CAUTION: The Pasteurellae are extremely dangerous pathogens, and strict aseptic technique must be maintained at all times. Handle cultures and specimens only under a bacteriological hood.

b. **Direct Examination.** In gram-stained smears of sputum, lymph fluid, or exudates from localized lesions, *Yersinia pestis* will appear as gram-negative, plump, ovoid bacilli exhibiting bipolar staining. *Francisella tularensis* usually will appear to coccoid forms in well-defined clusters. Either organism may possess capsules which are demonstrable by capsule staining. Cultural examinstion must be employed when plague or tularemia is suspected.

c. **Culture Examination.** *Y. pestis* may be isolated from blood using the routine methods described in chapter 2. *F. tularensis* is difficult to grow on routine medium and requires the addition of cystine or cysteine.

(1) *Trypticase Cystine Glucose Medium.* Use this medium for specimens other than blood for cultivation of *Yersinia pestis*. *F. tularensis* requires a cystine glucose blood agar. Several slants of this medium should be inoculated to increase the probability of isolation.

(2) *Special Media and Routine Media.* When plague or tularemia is suspected, it is imperative that special media be inoculated in addition to the routine media (blood agar and thioglycollate broth). This is necessary since other bacterial agents may cause systemic disease similar to tularemia or plague. Examine solid media for typical colonies suggestive of Pasteurellae. Prepare smears of these as well as any growth present in broth media. The tendency of bipolar staining of *Pasteurella* spp is better demonstrated by simple methylene blue, crystal violet, or dilute carbol-fuchsin stain. Table 9–5 shows differences between the *Yersinia* species.

9–23. Animal Virulence Test for Pasteurella (*Yersinia* and *Francisella*). Subcutaneous inoculation of a mouse or guinea pig with 2 to 3 ml of a 24-hour broth culture of *Y. pestis* will kill the animal within 2 to 5 days. Where *F. tularensis* is suspected, 2 to 3 ml of a saline suspension of a 48-hour should be inoculated subcutaneoulsy. In this care the animals die within 5 to 10 days. At necropsy, pasteurellae may be recovered from the spleen, blood, heart, lymphatics, and other tissues. Animal tests should be performed only in reference laboratories by qualified technicians exercising biologically controlled methods where spread of possible infection can be prevented.

Table 9–5. Identification and Differentiation of *Yersinia* spp.

Chapter 10
ACID-FAST BACILLI

10–1. Introduction:

a. Acid-fast bacilli are rod-shaped bacteria comprising the genus *Mycobacterium*. The term "acid-fast" refers to a staining characteristic of the microorganism and not to a specific taxonomic designation. Unlike most bacteria, these organisms stain with difficulty. Therefore, staining these organisms requires special techniques using heat or chemical energy to penetrate the cells with the dye. Once stained, the cells resist decolorization with acid-alcohol, giving rise to the term "acid-fast bacilli." Figure 10–1 shows an acid-fast stained smear of *M. tuberculosis* in sputum.

b. All of the acid-fast bacilli are presently found in part 17, Actinomycetes and Related Organisms, a group of Gram-positive rods that lie morphologically between the true bacteria and filamentous fungi. Two genera of the families Nocardiaceae and Actinomycetaceae—*Nocardia* (*N. asteroides*, and *N. farcinica*), and *Actinomyces* (*A. bovis* and *A. israelii*)—are mentioned here briefly because of their possible confusion with *Mycobacterium* spp. *Nocardia* are aerobic bacilli that grow well on routine media and stain somewhat acid-fast. *N. asteroides*, which is pathogenic, has been isolated from abscesses in man. More important, however, is its ability to cause tuberculosis-like disease in man.

c. Two species of *Actinomyces* are anaerobic, and nonacid-fast, but are of general interest in this section: *Actinomyces bovis*, which can be found in lesions sometimes observed on cattle and hogs; and *A. israelii*, whose habitat is in the mouth and tonsillar crypts of humans. The pathogenic actinomycetes *Nocardia* and *Actinomyces* are both recognized morphologically as gram-positive rods with beaded and branching shapes, but *Nocardia* are partially acid-fast and *Actinomyces* are nonacid-fast. Rapid growth on ordinary culture media distinguishes them from the tubercle bacilli.

10–2. Classification and Morphology:

a. **Taxonomy:**

Part 17. Actinomycetes and Related Organisms
Order—Actinomycetales
Family I. Actinomycetaceae
Genus 1—*Actinomyces*
Species—*bovis*
israelii
Family II. Mycobacteriaceae
Genus I—*Mycobacterium*
Species —*tuberculosis*
—*bovis*
—*avium*
—*leprae*
—*kansasii*
—*intracellulare*
—*fortuitum*
Family IV—Nocardiaceae
Genus I—*Nocardia*
Species— *asteroides*
—*farcinica*

b. **Morphology.** There are 30 plus species of mycobacteria which are morphologically similar; a general description of these organisms is provided. In tissues and exudates from infection, tubercle bacilli appear as small, thin rods with rounded ends. They may be straight or slightly curved and range between 2 to 4 μm in length and 0.3 to 1.5 μm in width. In culture smears, the longer, filamentous forms are occasionally observed, as well as smaller or club-shaped cells. Tubercle bacilli occur singly, in small groups, or occasionally in clumps of indiscernible cells. The irregular grouping of these forms is sometimes suggestive of branching (see figure 10–1). The leprosy bacilli and saprophytic acid-fast organisms closely resemble tubercle bacilli with regard to morphology as well as staining characteristics. Leprosy is usually differentiated from tuberculosis on a clinical basis but there are also cultural differences.

10–3. Cultural Characteristics. *M. tuberculosis*, *intracellulare*, *M. kansasii*, *M. avium* and *M. leprae* are presented below as primary organisms because they are the human pathogens most frequently encountered in the laboratory:

a. **M. tuberculosis.** The tubercle bacilli are aerobic and do not grow under strict anaerobic conditions. Cultures for isolation incubated at 35°C to 36°C show growth at 2 to 3 weeks on Lowenstein-Jensen media; at 12 to 15 days on 7H 10 medium. The growth is dry, friable, and nodular, with rough "cabbage head" centers. Colony growth on 7H 10 medium are rough and resemble a cauliflower in appearance. Whole colonies are easily detached from the agar but are difficult to suspend in saline due to their lipophilic properties.

b. **M. intracellulare.** *M. intracellulare* colo-

Figure 10–1. Acid-fast Stained Smear of *Mycobacterium tuberculosis* in sputum.

nies are characteristically thin, translucent, radially lobed to smooth, and cream-colored. Most strains show a small proportion of rough colonies, which may resemble colonies of tubercle bacilli.

c. **M. avium.** In the case of avian bacilli colonies form within 14 to 21 days. The colonies are smooth, viscid or butyrous, and hemispherical. They may possess a faint pink or yellow pigment. The growth is mucoid and tenacious, yet easily suspended in saline.

d. **M. kansasii.** *M. kansasii* bacilli colonies are generally smooth, although there is a tendency to develop roughness. They are cream colored when grown in the dark and become a bright lemon yellow if exposed to light. If colonies are grown in the light for 2 to 3 weeks, bright orange crystals of beta carotene will form on the surface.

e. **M. leprae.** *M. leprae*, also referred to as Hansen's bacillus, closely resembles the tubercle bacillus. It has not been successfully cultivated on artificial media, but can be propagated on the footpads of mice. These organisms are

characteristically seen in tissue or extracellularly, arranged in groups or packets, side by side.

10-4. Pathogenicity. There are many species of *Mycobacterium;* those species pathogenic to man (always or sometimes) other than *M. tuberculosis* and *M. bovis* are Runyon Group I (*M. Kansasii* and *M. marinum*); Group II (*M. scrofulaceum*); Group III (*M. avium, M. intracellulare,* and *M. xenope*); and Group IV (*M. fortuitum* and *M. chelonei*). The pathogenicity of *M. tuberculosis, M. intracellulare, M. avium, M. kansasii,* and *M. leprae* will be discussed in this section.

a. **Mycobacterium tuberculosis.** *M. tuberculosis* is one of the causative agents of tuberculosis in man. The organism may gain entry into the body by way of the respiratory or alimentary tracts or through the conjunctiva of the eye. Infected individuals, in the processes of sneezing, coughing, or expectorating, produce an infectious aerosol containing droplets and contaminated dust particles, which may be inhaled by susceptible individuals. Infections also are acquired from fomites (towels, drinking cups, clothing, etc). Following the initial infection, tubercle bacilli form primary and secondary lesions within the tissue. The organism may then spread to various tissues via the lymphatic system or the bloodstream. Invasion of the bloodstream results in the bacilli's dispersion throughout the body, giving rise to acute miliary or chronic disseminated tuberculosis. Practically any tissue of the body is subject to invasion by tubercle bacilli. However, more than 90 percent of deaths from tuberculosis are due to pulmonary infection. Infections of the bone, bone-joints, lymph nodes, spleen, liver, kidney, meninges, and gastrointestinal tract occur, but with lower percentage. The disseminated infections are somewhat more prevalent in children. Lesions formed by pathogenic acid-fast bacilli are referred to as tubercles. Tubercles may either rupture and discharge their bacilli to produce further infection, or they may seal and permanently wall off the bacilli by fibrosis or calcification. By surviving the first infection, man or animal may acquire some resistance (cellular immunity) to tuberculosis. Upon subsequent infections, the defense mechanisms of these hosts have an increased capacity to localize and destroy tubercle bacilli. Although antibodies are formed by the host against a variety of cellular antigens within the tubercle bacilli, such antibodies appear to be of little value in increasing resistance. The increased resistance to infection is largely attributed to the mononuclear cellse, which acquire a greater ability to ingest tubercle bacilli. Mononuclear cells develop this property in the course of primary infections. If virulent tubercle bacilli are injected subcutaneously into a guinea-pig, it develops a local lesion during the next few weeks, with extension of the infection first to the local lymph nodes and then throughout the body. A second injection given a few days after the first injection produces similar results. But if more than 2 weeks elapse between the first and second injections, the latter provokes a vigorous local inflammatory response which begins within a day or two and often culminates is sloughing of the skin and subcutaneous tissues at the site of injection. Regional lymph nodes are generally not involved. This alteration of reactivity and host's acquired resistance is called Koch's phenomenon, after its discoverer. Koch also observed that subsequent to the primary infections, the hypersensitivity reaction could be elicited by injecting dead bacilli, or even "old tuberculin," a bacterium-free concentrate of broth in which tubercle bacilli had been grown. No local response follows the subcutaneous injection of either dead tubercle bacilli or old tuberculin into a guinea-pig which has no previous experience of tuberculosis. This phenomenon of the hypersensitivity response stimultaed by the tuberculosis infection is of value in the diagnosis of "tuberculosis" as it forms the basis of the tuberculin skin test.

b. **M. intracellulare.** *M. intracellulare* bacilli are responsible for a pulmonary disease indistinguishable from that caused by *M. tuberculosis* and *M. kansasii,* although it is not isolated as frequently. Patients from whom this organism is isolated give a history of exposure to poultry, swine, or other animals. The organisms respond poorly, if at all, to drugs.

c. **Mycobacterium avium.** *M. avium,* the causative agent of avian tuberculosis, is found in wild birds and domesticated fowl and from time to time appears in human infections.

d. **M. kansasii.** *M. kansasii* is responsible for a severe pulmonary disease in man, most frequently appearing in white, emphysematous males over the age of 45 years. The disease with its complications is indistisguishable from that caused by *M. tuberculosis.* It is more chronic, but less communicable than *M. tuberculosis. M. kansasii* has been isolated frequently in the midwestern part of the United States, but this

does not rule out its presence in other parts of the country.

e. **Mycobacterium leprae.** *M. leprae* is the causative agent in leprosy, an ancient disease known as early as 1400 B.C. Leprosy still occurs in certain Asiatic countries, in South America, and to a limited extent in the southern United States. Isolated and identified in 1878, the organism spreads mainly by personal contact, but exposure over a period of years seems necessary for infection to take place. Details of the actual mechanism of transmission organisms are defined as those that live on dead are still lacking.

f. **Saprophytic Mycobacteria.** Saprophytic organic matter. Some normally saprophytic species can occasionally invade the tissues of living animals and humans, but this is rare. Saprophytic mycobacteria frequently occur in clinical specimens and must be differentiated from pathogesic forms. These include such species as *Mycobacteria smegmatis, M. phlei,* and *M. butyricum.*

10–5. Growth Requirements:

a. **Mycobacterium tuberculosis.** *M. tuberculosis* requires an optimum temperature of 36°C to 37°C. Media containing homogenized whole egg, glycerol, asparagine, and potato starch have been used successfully in the cultivation of the organism because they contain the previously mentioned constituents. Malachite green also is incorporated into each medium to inhibit growth of organisms other than mycobacteria. Middlebrook 7H10 agar with oleic acid, dextrose, and citrate enrichment has been added to the arsenal of diagnostic media. It is an excellent medium to use for primary isolation and susceptibility testing of *M. tuberculosis.* This medium has several other advantages. Early detection of colonies with the aid of the microscope is possible because the medium is clear. It permits easy separation of mixed colony types, and contamination with other bacteria or fungi does not alter or destroy the agar base medium as quickly as it does the egg base media. It is an excellent medium for demonstrating "cording". Middlebrook's medium shows growth in 2 to 3 weeks as compared to 3 to 6 weeks for an egg base medium. Middlebrook 7H10 must be relatively fresh, however, and cultures must be incubated under increased CO_2 tension (5 to 10 percent). It is important to note that CO_2 tession is a stimulant to growth of the tubercule bacillus in both Middlebrook and egg medium.

b. **M. intracellulare.** *M. intracellulare* bacilli grow slowly at 36°C (10 to 21 days) and 25°C on Tween 80 or tellurite media.

c. **M. avium.** *M. avium* requires a temperature of 40°C to 42°C for about 2 to 6 weeks before evidence of growth is detected.

d. **M. kansasii.** Optimal growth of *M. kansasii* occurs after 2 to 3 weeks at 36°C (slower at 25°C); growth does not occur at 45°C.

e. **M. leprae.** *M. leprae's* growth requirements have not been determined at this writing.

10–6. Identification. The identification of Mycobacterium isolates rests partly on the comparison of several characteristics which already have been covered and which are summarized in table 10—the temperature range for growth and the period required for colony formation—and partly on additional characteristics, such as the effect of bright light on pigment production, and certain biochemical procedures are useful in differentiating the various species of mycobacteria. These are discussed in this section, paragraph e. As a further aid to identification, laboratories equipped to perform animal studies can confirm the pathogenicity of isolates bf inoculating guinea pigs, rabbits, and mice. CAUTION: All clinical and laboratory materials suspected of being contaminated with pathogenic mycobacteria or related species should be handled under a hood with strict aseptic technique. These organisms are highly resistant to adverse environmental conditions. When flaming the wire of the inoculating needle, "spattering" of the specimen must be avoided. The use of a bacteriological hood is highly desirable at all times, but especially when extensive work with acid-fast organisms (AFB specimens) is undertaken.

a. **Culture Specimens.** The specimens examined for diagnosis of tuberculosis may consist of a variety of materials depending upon clinical manifestations. Other mycobacteria may be encountered when looking for the tubercle bacilli. Sputum, urine, and gastric washings are most often analyzed, but tissue specimens, lymph aspirations, and pleural, pericardial, spinal, or joint fluids may be submitted upon occasion. As the study of tuberculosis is a specialty in the true meaning of the word, some of the various laboratory tests employed in this field and their significance are not covered by this manual. The reader should consult current standard reference books and the cytochemical tests for acid-fast bacilli described in chapter 14 of this manual.

b. **Staining.** Mycobacteria stain poorly with the gram stain. Technically they cannot be

Table 10–1. Key Differential Features for Clinically Significant Mycobacteria.

	Opt. Temp.	Rate of Growth	Pigment	Guinea pig Virulence
M. *tuberculosis*	37° C	Eugonic	Positive	Positive
M. *bovine*	37° C	Dysgonic	Negative	Positive
M. *avian*	40–42° C	Rapid	Positive	Variable*
Atypical AFB	25–37° C	Rapid	Positive	Negative

*Positive virulence in chicken.

classified either as gram-positive or gram negative. This is because large amounts of liquids, fatty acids, and waxes within the cells impede penetration of the dye. Staining difficulties are overcome when special methods are employed, such as the Ziehl-Neelsen, Kinyoun techniques Auramine "O" or Auramine-Rhodamine Fluorescent staining procedure described in chapter 3. Tubercle bacilli often appear as irregularly stained forms, exhibiting a banded or beaded appearance.

c. **Direct Examination:**

(1) *Smears.* Smears should be prepared on all clinical specimens from possible tuberculosis and stained by one of the techniques previously described. A positive acid-fast stain constitutes only presumptive evidence of tuberculosis. The final decision as to whether or not the organism is a pathogen is determined by careful cultural studies and animal virulence tests. Concentration of the specimen prior to staining markedly improves chances of detecting mycobacteria if present in either small or large quastity. Specimens with negative results for AFB smears should be concentrated, smeared, stained, and reexamined again.

(2) *Reporting Format.* The results of microscopic examination of direct smears from clinical material are often reported as positive or negative for acid-fast bacilli. However, the National Tuberculosis Association (NTA) suggests the following reporting format:

(a) "Rare"—three to nine organisms per slide.

(b) "Few"—10 or more organisms per slide.

(c) "Numerous"—10 or more organisms in most oil immersion fields observed.

(d) If fewer than three positive fields are found, prepare a new slide.

d. **Pigment Production—the Acid-Fast Chromogens.** The acid-fast bacilli," as classified by Runyon (table 10–2). Runyon separated the medically significant strains of unclassified mycobacteria into four groups as a rough classification, until a more precise placement could be determined. Recent techniques using metabolic and biochemical testing have enabled workers to place the mycobacteria into the groups suggested earlier by Runyon. Runyon's groups are as follows:

(1) *Group I, Photochromogens.* The photochromogens can produce serious pulmonary disease in man. The organisms form a lemon-yellow pigment is the presence of light; hence, the name "photochromogen." *M. kansasii*, commonly called the "yellow bacillus," is the most frequent isolate from this group.

(2) *Group II, Scotochromogens.* This group is exemplified by *M. scrofulaceum*, which produces a yellow or orange to dark red pigment whether cultivated in the light or in darkness. Not generally considered to be pathogenic, this organism has nevertheless been associated with cases of rare pulmonary disease and lymphadesitis in children.

(3) *Group III, Nonphotochromogens.* The nonphotochromogens, often referred to as the "Battey bacilli," cause a form of tuberculosis clinically indistinguishable from the pulmonary infections produced by *M. tuberculosis* and *M. kansasii*. Unlike the true tubercle bacillus, the Battery strain often shows a primary drug resistance that makes treatment difficult.

(4) *Group IV, the Rapid Growers.* The rapid growers of the Runyon scheme are so labeled because growth occurs in 3 to 4 days instead of 2 to 3 weeks as is characteristic of the other mycobacteria. Most of the rapidly growing potentially pathogenic isolates from sputum, abscesses, and other clinical specimens are *M. fortuitum*. The saprophytes *M. smegmatis* and *M. phlei* are often isolated from clinical specimens. Because these species are rapid growers, they are implicated in this group, but neither of them has been suggested in a disease process. *M. smegmatis*, commonly referred to as the "smegma bacillus," is found

occasionally is urine specimens. This bacillus is very difficult to distinguish microscopically from the pathogenic acid-fast bacilli. The organism is widely distributed in water, soil, and dust and can be isolated from human smegma. *M. phlei* also is found in the soil in dust, and on plants. Commonly called the "timothy grass bacillus," the organisms form soft, waxy colonies with dark yellow pigmentations.

e. **Biochemical Procedures.** Several biochemical procedures are useful is the differentiation of species in the Genus *Mycobacterium* are discussed in the following paragraphs; they are the niacin test, the nitrate reduction, the calalase activity test, the aryl sulfatase test, Tween 80 hydrolysis and sodium chloride (NaCl) tolerance test. A summary of these procedures is contained in table 10–2.

(1) *Niacin Test.* Niacin production is detected by a color reaction with cyanogen bromide and aniline.

(a) Pipette 1.0 ml of sterile saline to a 3-week or older culture slant. (Be sure there is no niacin-containing additive, such as penicillin, in the medium.)

(b) Allow the saline to remain for 30 minutes.

(c) Transfer a portion of the saline to a small screw-capped test tube.

(d) Add as equal portion of aniline and cyanogen bromide solution to the saline extract.

(e) If niacin is present, a yellow color will appear instantly. CAUTION: Since cyanogen bromide is a tear gas, the test should be performed in a well-ventilated area or under a fume hood.

(f) Positive and negative reactions are shown in table 10–1.

(2) *Nitrate Reductions.* This test is useful in differentiating slower growing organisms (*M. tuberculosis* and *M. kansasii*) from member of Group II mycobacteria and the clinically important Battey-avian organisms. The former are strongly positive nitrate producers, whereas the Battey-avians are generally negative. See chapter 14, paragraph 28 for this test procedure.

(3) *Catalase Activity Test.* All acid-fast bacilli produce catalase, but there is a decrease in catalase production as the bacilli become resistant to the threpeutic agent, isoniazid. This decrease is correlated with a weakening of virulence of the organism against the guinea pig. It is possible to subgroup the acid-fast bacilli on the basis of their catalase activity at different temperatures and pH. At a temperature of 68 C and a pH of 7.0, the catalase of human and bovine tubercle bacilli is selectively inactivated. Under the same conditions, all other acid-fast species are catalase positive. The procedures for checking catalase activity are as follows:

(a) Prepare a 1:1 mixture of 10 percent Tween 80 and 30-percent hydorgen peroxide. Both should be at room temperature.

(b) Add 0.5 ml of the mixture to the Lowenstein-Jensen slant. Bubbling indicates a positive catalase test.

(c) To test catalase activity at 68°C, add several loops of mycobacteria from a slant to 0.5 ml of a phosphate buffer solution (pH 7.0) in a test tube.

(d) Incubate this mixture in a waterbath at 68°C for 30 minutes.

(e) Add 0.5 ml of the Tween-peroxide mixture to the buffered growth solution.

(f) Observe for bubbling, which indicates a positive reaction.

(4) *Aryl Sulfatase Test.* The aryl sulfatase test measures the ability of mycobacteria to form an enzyme—sulfatase—whes grown in a medium containing 0.001 molar tripotassium phenolphthalein disulfate. The enzyme, if present, liberates phenolphthalein, which is detected by adding an alkali to develop a characteristic red color. The procedures for performing the test are as follows:

(a) Inoculate a portion of an active growth from a Lowenstein-Jenses culture to Wayne's phenolphthalein agar by surface streaking and stabbing.

(b) Incubate for 72 hours at 37°C.

(c) Add 0.5 ml of 1 M sodium carbonate solution to both the inoculated medium and an uninoculated control medium.

(d) Formation of a pink color is the agar or the supernatant fluid indicates a positive reaction.

NOTE: The atypical species are sulfatase-positive; the avian, bovine, and human species are negative. If positive, check table 10–1 to determine the species of the organism.

(5) *Tween 80 hydrolysis.* Tween 80 can be hydrolyzed by certain species of mycobacteria to produce oleic acid (a derivative of sorbitan monoleate). A pink to amber color is obtained with the use of neutral red as the pH indicator. The indicator itself (reddish at acid pH) is changed to amber in the substrate and goes back to a pink or red color in positive reactions. The test is performed as follows:

(a) One loopful (3 mm) of an actively growing L-J culture is placed into a tube containing Tween 80 substrate (m/15 phosphate

Table 10-2. Biochemical (4-Test) Summary for Clinical *Mycobacteria* spp.

Species or Subgroup	Niacin Production	Nitrate Reduction		Catalase Produced (mm)			Tween Hydrolysis (Days)		Tellurite Reduction in 3 days	Pigment Formation		Growth on 5 percent NaCL	Growth in less than 7 days at 37°C	Produce Arylsulfatase enzyme +3 days	Growth on MacConkey Agar
		>1+	<3	>40	>50	68+	+5	+10		Dark	Light				
Properties of Mycobacteria Species															
*M. tuberculosis	A	A	A	A	E	E	C	C	E	E	E	E	E	E	E
M. bovis	E	E	E	A	E	E	E	E	E	E	E	E	F	E	F
Runyon Group I (Photochromogenic) (lemon-yellow pigment)															
*M. kunsasii	E	A	A	A	A	A	A	A	E	E	A	E	F	F	E
*M. marinum	E	E	E	C	D	E	A	A	E	E	A	-	D (32°)	E	E
Runyon Group II (Scotochromogenic) (yellow-orange to dark red)															
M. scrofulacrum	E	D	E	A	A	A	E	E	E	A	A	E	E	E	E
M. aquae	E	E	E	A	A	A	B	A	E	A	A	E	E	E	E
M. florescens	E	A	B	A	A	A	A	A	E	A	A	C	E	E	E
Runyon Group III (no pigments in light)															
*M. avium	E	E	E	A	E	A	E	E	C	E	D	E	E (41°)	E	E
*M. intracellulare	E	E	E	A	E	A	E	E	B	E	E	E	E (41°)	E	E
M. xenopei	E	E	E	A	E	A	F	E	E	D*	D*	E	E	C	E
M. gastri	E	E	E	A	E	E	A	A	E	E	E	E	E	E	E
Runyon Group IV (rapid growers) (some scotochromogens)															
M. fortuitum	E	C	C	A	A	A	E	D	A	E	E	A	A	A	A
M. smegmatis	E	A	D	A	A	A	A	A	A	E	E	A	A	E	E
M. phlei	E	A	D	A	A	A	A	A	A	A	A	A	A	E	E

A = 85% or more
B = 75-84%
C = 50-74%
D = 15-49%
E = <15%

*Pigment increases with age.

buffer, pH 7, 100 ml containing 0.5 ml tween 80 and 2.0 ml of 0.1 percent aqueous neutral red) and incubate this at 35°C.

(b) Set up a control tube using *M. kansasii* (a known positive) along with an uninoculated (negative control) tube. The tubes are allowed to incubate for 5 days prior to testing, and replaced in incubator if no reaction is noted.

(c) On the 10th day of incubation the tubes are again inspected for a color change.

(6) *Sodium Chloride Tolerance Test.* The procedure is useful generally in distinguishing the rapid-growing mycobacteria (positive) from the slow growers (negative), also to aid in the identification of *M. fluorescens* (sometimes positive). The medium is prepared by adding sodium chloride (NaCl) in a final concentration of 5 percent to the Lowenstein-Jensen slants used. A light suspension containing the organism is prepared and a 1 ml is inoculated to the surface of NaCl medium and a control slant containing no NaCl. The slants are incubated at 35°C and inspected weekly for 4-weeks growth on both slants is a positive salt tolerance test.

f. **Guinea Pig Virulence Test.** The presence of acid-fast bacilli in direct examination and of typical colonies on agar is strong evidence of tuberculosis. Demonstration of specific lesions in laboratory animals is generally not necessary but useful in confirmation. The guinea pig virulence test is one method of providing this information. Many clinical laboratories are not equipped to perform animal pathogenicity tests. When facilities are lacking, cultures should be shipped to reference laboratories for confirmation of identification. Specimens to be shipped for culture should be placed in equal volumes of trisodium phosphate solution. The guinea pig virulence test is performed as follows:

(1) Prepare an approximate 2-ml volume of a heavy saline suspension of specimen concentrate or organisms from a typical colony.

(2) Select a previously skin-tested, tuberculin-negative guinea pig weighing at least 200 gm.

(3) Inject the 2-ml suspension intraperitoneally, being careful not to puncture the intestines.

(4) The injection site will usually develop into a local tuberculous lesion before a generalized infection is noted.

(5) Weigh the animal at weekly intervals. Persistent weight loss is usually the first sign of infection.

(6) Perform a skin test in the fourth week or when lymphatic enlargement occurs.

(7) If the skin test is positive, the animal should be sacrificed and autopsied. If the skin test is negative, hold the animal for 4 more weeks, sacrifice it, and perform an autopsy.

(8) Examine for caseous lesions at common sites, such as the spleen, lymph nodes, liver, kidneys, lungs, and the inoculation site. Smears are prepared from tissues for examination.

(a) Transfer a portion of the caseous material (sputum-bloody purulence) to a precleaned slide.

(b) Press another slide onto material and squeeze the slides together, then pull them apart. Make sure materials are not so thick on the slides that they give a stain too dark for examisation.

(c) Label slides and air dry, flame immediately two or three times.

(d) Stain according to the Ziehl-Neelsen of Kinyoun acid-fast methods.

(e) Dry in air, do not blot.

(f) Examine under oil immersion lens.

(9) Dissect tissues and prepare slides by pressing the materials onto a slide; heat fix for staining according to the Ziehl-Neelsen method.

(10) A diagnosis of tuberculosis has such serious implications for the patient that it is important to prove beyond any doubt the nature of the organism isolated.

g. **N-acetyl-L-Cystine Method Solutions.** (Digestion and decontamination of sputum for mycobacterium tuberculosis) Kubica et al.

(1) Prepare a volume of digestant by adding equal volumes of 1N (4 percent) sodium hydroxide and 0.1 M (2.94 percent) sodium citrate · $2H_2O$ adding 0.25 gm of N-acetyl-L-cysteine powder for every 50 ml of combined solution, that is, 25 ml + 25 ml + 0.25 gms. The solution is self-sterilizing but deteriorates rapidly, and should therefore, be used within 24 hours.

(2) In a well-ventilated area (safety hood) transfer about 10 ml (or slightly less) of sputum or bronchial secretion to a sterile 50-ml, screwcapped, aerosol-free centrifuge tube. Smaller volumes may be used in small tubes, but in no case should the volume of the specimen exceed one-fifth the volume of the tube.

(3) Add an equivalent amount of the NALC digest to the specimen, mix well with a tube mixer (Vortex). Digestion is generally effected in 5 to 30 seconds. Avoid extreme agitation, which may inactivate the NALC by

oxidizing it. Continue with procedure as stated below (h3).

h. **Concentration of Sputum Specimens.** Concentration of sputum specimens may be accomplished by digesting the samples with sodium hypochlorite. Under these conditions, any mycobacteria present are concentrated upon centrifrigation of the specimen. Centrifugation will generally increase the number of positive smears. The sediment obtained from digestion should not be used for inoculation of either cultures or test animals for the reason that sodium hypochlorite is tuberculocidal. The procedures for digestion and concentrations are as follows:

(1) Mix equal volume of sputum and 5 percent sodium hypochlorite (household bleach) in a collecting container (50-ml size); stir with a wooden applicator stick to ensure mixing of materials.

(2) Shake materials for 2 to 3 minutes, keeping mixture at room temperature for 10 minutes or until digestion is complete.

(3) Transfer a portion of sputum to a 15-ml centrifuge tube and centrifuge for 10 minutes at 3000 r/mis.

(4) Pour off supernatant fluid and allow the tube to stand in an inverted position on an absorbent material (paper towel or filter paper in order to drain the sediment (neutralization is not required).

(5) Transfer the creamy white sediment to a slide with a cotton-tipped applicator stick. Air dry (so fixing is required) and stain by the agent in sputum cultures for tuberculosis is Ziehl-Neelsen or Kinyoun method.

i. **N-acetyl-L cysteine sodium hydroxide Culture Method**. The use of NALC as a mucolytic recommended as a mild decontaminant and digestion procedure.

(1) Collect the sputum (an early-morning, deep-cough specimen) and transfer approximately 10 ml to a 50-ml, sterile, disposable, conical, screw capped tube.

(2) Add an equal volume of NALC (with *sodium hydroxide* solution).

(3) Tighten screw caps and mix well in a mixer (vortex) for 10 to 20 seconds to complete digestion. Gently agitate the mixer, because violent mixing can cause the NALC to deteriorate.

(4) Allow to stand at room temperature for 15 minutes so that decontaminating effects of solution can take place.

(5) Fill the tube within one-half inch of the top with Sorensen buffer, pH 6.8, or sterile distilled water.

(6) Centrifuge at 3000 r/min for 15 misutes and carefully decant the supernatant fluid into a vessel containing a phenol disinfectant. Wipe the mouth of the tube with a cotton ball soaked with 5 percent phenol. Keep the sediment.

(7) Make a smear using a sterile, cotton-tipped applicator stick or a wire loop. Spread the sediment on an area about the size of a penny. Stain by the Ziehl-Neelsen method and determine the approximate number of AFB (acid-fast-bacilli) per oil-immersion field.

(8) Add 1 ml of 0.2 percent bovine albumin to the sediment using a sterile pipet. Mix with gentle shaking, (neutralization is not required due to phosphate buffer wash, step 5). Refrigerate the sediment overnight, if not pressed for time. It is recommended that the albumin be added before the smears are made if the volume of sediment is small.

(9) Make a 1:10 dilution of this sediment by adding 10 drops of it to 4.5 ml of sterile water.

(10) Inoculate the proper media, using 0.1 ml of the sediment for each plate. Plate two tubes using undiluted and diluted sediment preparations for incubation at 35°C to 37°C. Consult paragraph 10–6 for identification of cultured colonies.

Chapter 11
SPIROCHETES

11–1. Introduction. The spirochetes are protozoan-like members of the order Spirochaetales. Spirochetes were among the earliest microorganisms to be seen under a crude magnification. The term "spirochete" includes a host of slender, flexible, filamentous, corkscrewlike organisms, which are found distributed widely in nature. They are found in water, soil, plants, animals, and even in man. Most of these microorganisms are soprophytic, but a few are pathogenic. Those pathogenic members are the concern of this chapter because they cause such diseases as yaws, relapsing fever, syphilis, and also certain hemorrhagic disorders.

11–2. Classification and Morphology:

a. **Taxonomy.** Three genera included in the order Spirochaetales are pathogenic for man:

Part 5: Spirochetes
Order: Spirochaetales
Family I. Spirochaetaceae
Genus III—*Treponema*
Species—*pallidum, pertenue, microdentium*
Genus IV—*Borrelia*
Species—*recurrentis, vincentii*
Genus V—*Leptospira*
Species—*icterohemorrhagiae canicola, pomona*

b. **Morphology:**

(1) *Borrelia spp. Borrelia* is a spiral threadlike organism measuring 0.2 to 0.5 μm in width and 8 to 16 μm in length, and possessing from 4 to 10 spirals, with most organisms having 5. The spirals are large and wavy with a distance of approximately 2.5 μm between them. Amplitude is about 1.5 μm, as shown in figure 11–1. Occasionally, axial terminal filaments are seen, and these may give the appearance of flagella. The spirochetes can be stained with the gram stain, in which they appear gram-negative. The Romanowsky modification or Giemsa-stain, which stains the organism purple, also can be used. The mechanics of staining cause some distortion and straightening of the spirals. On this point, experience must be gained if this stain is to be used in generic identification. *Borrelia* can be viewed under darkfield conditions in a wet-mount preparation. Under these conditions, the actively motile spirochetes have a corkscrew movement along with a side-to-side motion. This motion is not necessarily directional, and the organism may be seen to move back and forth.

(2) *Treponema spp. Treponema* occur as very fine, delicate spiral forms having a flexible, cylindrical body measuring approximately 0.25 μm in diameter and 5 to 16 μm is length. The spiral amplitude is approximately 1.0 μm. The organism possesses from 8 to 15 regular spirals. Figure 11–2 is a drawing from a photomicrograph of two *Treponema pallidum* organisms, seen touching at one end. Motility is generally slow, although the organism rotates rapidly on its axis. During the rotation process, the organism bends to form circular as well as "*S*" shapes. In materials aspirated from syphilitic lesions in the early stage of infection, the organism tends to be elongated (less spiraled), and movement is snake-like. This is due to the greater density of the mucoid fluid present in the lesion.

(3) *Leptospira spp. Leptospira* measure approximately 0.15 mm in diameter and 7.0 to 20. μm in length. There are generally 12 to 18 spirals in each organism, having an amplitude of 0.4 to 0.5 μm. Characteristically peculiar to *Leptospira*, the third terminal of the organism is quite flexible and is often seen in the shape of a hook. This is illustrated in figure 11–3.

11–3. Growth Requirements:

a. **Borrelia spp.** *Borrelia* spirochetes can be artificially cultivated in liquid media, such as ascitic fluid containing a small amount of rabbit kidney along with several drops of citrated blood and having a final pH of 7.2 to 7.4.

b. **Treponema spp.** Pathogenic treponemes have not been cultivated in the laboratory. However, the Reiter strain of *Treponema pallidum* has been grown under certain conditions in thioglycollate or other anaerobic broths containing serum. Attempts to culture the treponemes in tissue or fertile eggs have failed; therefore, cultivation is not part of the diagnosis.

c. **Leptospira spp.** *Leptospira* can be grown under laboratory conditions. Culturally, the leptospiral forms are aerobic and grow best at a temperature of 25°C to 30°C. *L. icterohemorrhagiae* and some of the other species can be easily cultivated in liquid medium containing 10 percent rabbit serum, 0.2 percent agar, and a small amount of hemoglobin dispensed in physiological saline. Some species of

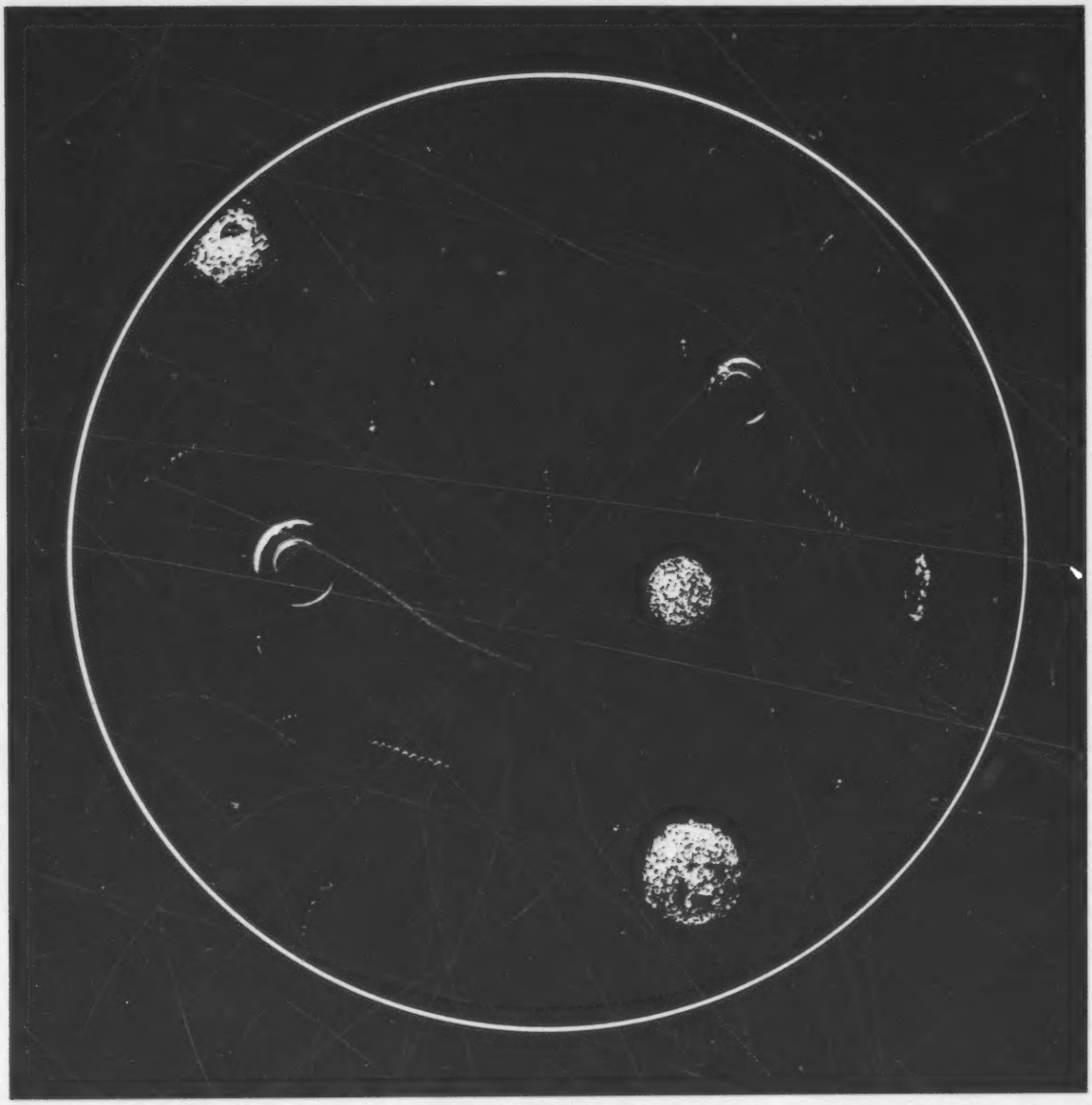


Figure 11–1. Darkfield Mount of *Treponema pallidum* in Exudate From a Penile Lesion.

Leptospira will grow without the presence of the rabbit serum. Colonies are rarely seen on the surface of agar plates.

11–4. Pathogenicity:

a. **Borrelia spp.** There are a number of different species of *Borrelia*, many of them very similar in morphology; however, only two of these will be discussed here—*Borrelia recurrentis* and *Borrelia vincentii*.

(1) *B. recurrentis* is the causative agent in relapsing fever, an ancient disease described some 20 centuries ago by Hippocrates. Although the disease is still prevalent in Central America, India, Africa, and parts of Europe, it has not been observed in epidemic form in the United States. The organism is transmitted from man to man by body louse and from rodent to man by ticks. A high fever develops within 3 to 10 days following infection; there may be numerous organisms in the bloodstream and on occasion in the urine as well. After 4 to 5 days, the fever subsides and the number of organisms in the bloodstream de-

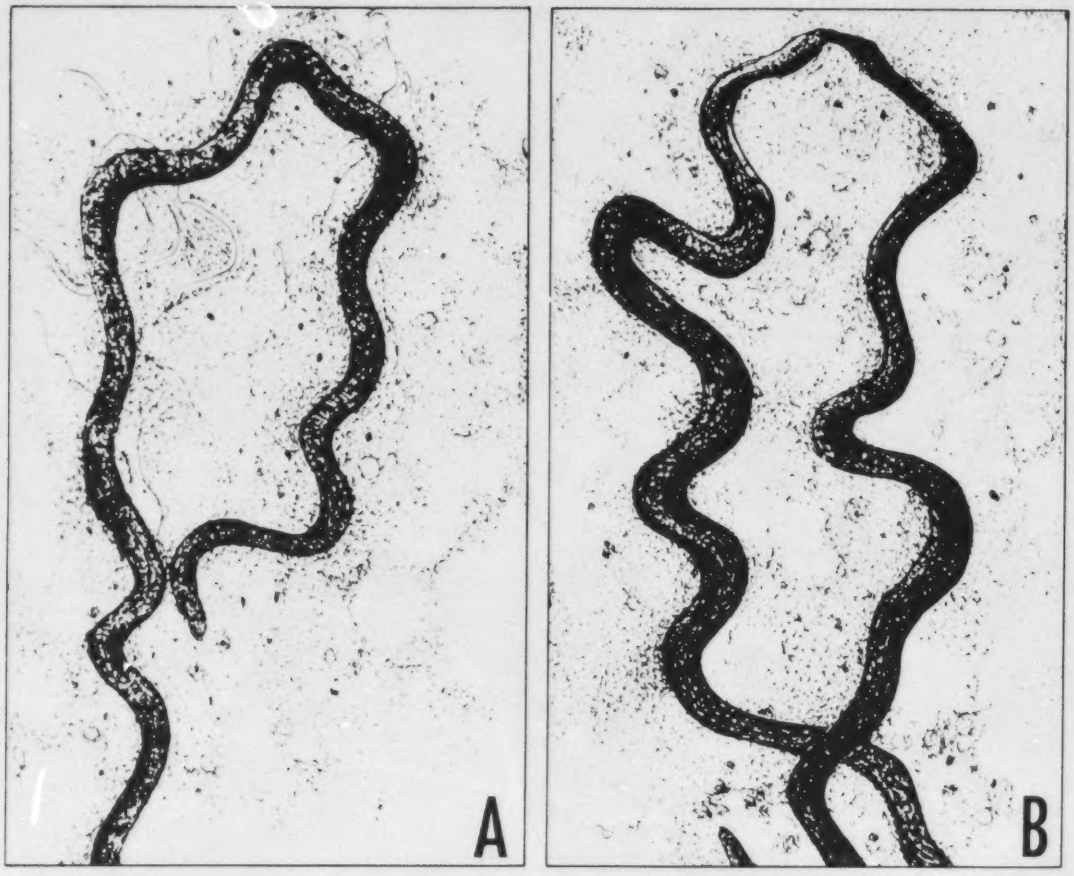


Figure 11–2. Photomicrograph of Two *Treponema pallidum* organisms.

creases. Those organisms remaining take on rather atypical and bizarre forms and lose their motility. This period of low grade fever or normal temperature may last for 1 to 4 weeks before the occurrence of another febrile attack. At this time organisms may invade the spleen, liver, gastrointestinal tract, and the kidney.

(2) *B. vincentii*, in combination with the fusiform bacilli, is the causative agent of Vincent's angina. The disease may also be referred to as a fusospirochetal infection or trench mouth. This condition is characterized by buccal ulcers and throat sores.

b. **Treponema spp.** The treponemes are perhaps the most widely known of the spirochetes via knowledge of syphilis. However, other species are considered here along with *T. pallidum;* they are *T. pertenue*, *T. microdentium*, *T. mucosum*, and *T. genitalis.*

(1) *T. pallidum* causes the venereal disease syphilis in man. The organism enters minute breaks in the skin or mucous membranes to infect the individual sometime during sexual activity with an infected partner. The spirochetes remain at the site of initial infection for a time multiplying in number. Infection also may be transmitted by indirect means, towels or drinking receptacles, for example. The spirochete is very fragile, however, and it is unlikely that it can survive on artificial surfaces long enough to cause infection. The characteristic reaction following infection is an inflammatory response known as the chancre. This stage is referred to as primary syphilis and the chancre forms within 10 to 90 days following infection. The chancre lasts from 1 to 3 weeks and may heal spontaneously. Within 2 to 12 weeks after the primary chancre heals, a generalized skin rash usually appears. This stage of the disease is referred to as "secondary syphilis." Symptoms usually disappear within about 3 weeks but in untreated cases may reappear one or more times as relapses.

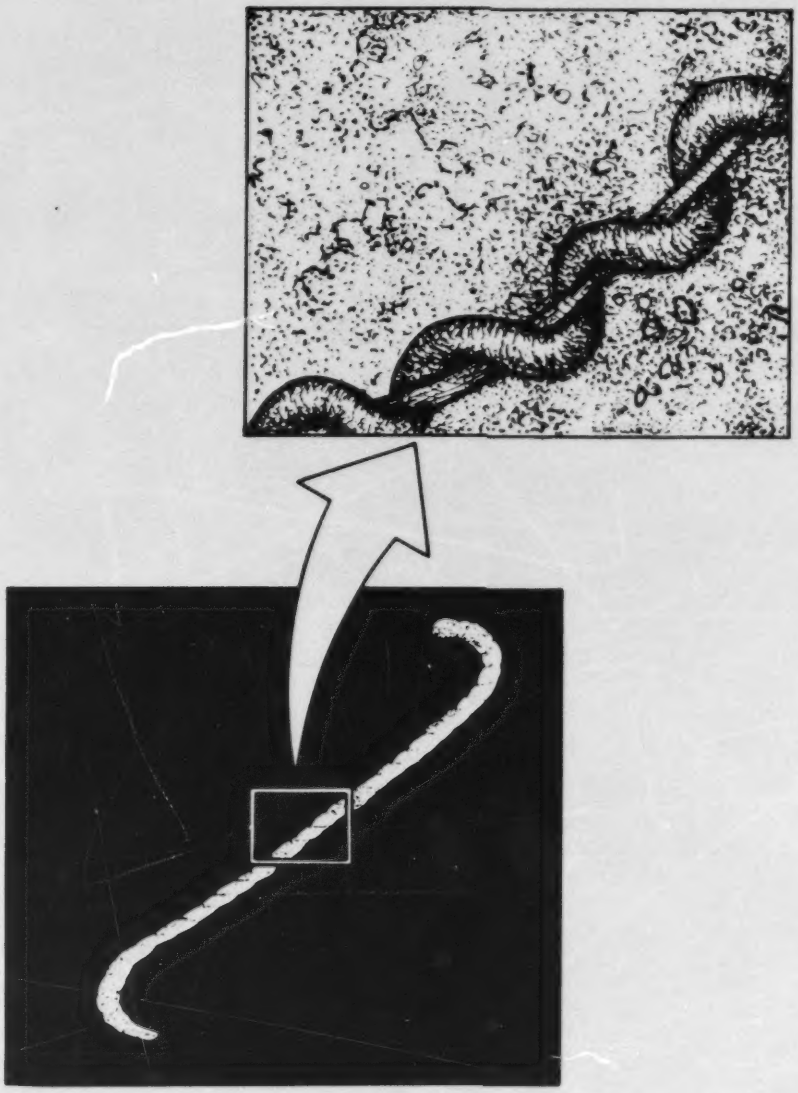


Figure 11–3. *Leptospira.*

In secondary syphilis the characteristic skin lesions contain *Treponema pallidum.* An increasingly positive serologic test is the rule. The subsequent latent stage of syphilis is usually shown by a persistently positive reaction in serologic tests. Late symptomatic syphilis, which can be expected to follow the latent period in untreated cases, also can be detected by reactive serologic test.

(2) *T. pertenue* is the causative agent of an ulcerative skin disease of the tropics called yaws. The disease is prevalent in those tropical regions where rainfall is heavy. It is transmitted to man by *Musca spectanda* (a species of fly) coming in contact with an open lesion. Yaws starts with a primary lesion and in 1½ to 3 months secondary lesions, which are deeper in nature, appear. Later on in the disease, there may be bone involvement.

(3) *T. microdentium* and *T. mucosum* are found in the mouth; the latter is associated with gum disease and pyorrhea alveolaris.

(4) *T. genitalis* has been implicated in venereal disease, but without definite proof.

c. **Leptospira spp.** Pathogenic leptospiras are generally parasites of lower animals both domesticated and nondomesticated. Man is infected through direct contact with infected

tissues or excreta from a diseased animal. *L. icterohemorrhagiae* is the prime organism of concern in this genera.

(1) *L. icterohemorrhagiae* is the causative agent in hemorrhagic jaundice (Weil's disease), a disorder encountered worldwide. In the United States it is only occasionaliy seen, and only about half of the persons infected develop the typical clinical picture of jaundice. After infection the leptospira are distributed throughout the body, and in later weeks are found in the urine.

(2) *L. canicola* causes a nonjaundice type of leptospirosis found predominantly in animals—dogs, pigs, cattle, sheep, and horses. The disease is known as Stuttgart disease and is similar to Weil's disease in man. It is rather common in dogs and is transmitted to man. This disease is less severe than Weil's disease and is usually missed because there is no telltale jaundice signaling infection. (Species not recorded in Bergy's 1974.)

11–15. Identification:

a. **Darkfield Attachment.** The darkfield apparatus is the single most important tool in the identification of members of Spirochaetales. Routinely used microscopes may be converted to darkfield prior to each use or a separate microscope setup requiring only minor adjustments can be maintained ready for use at any time. In this case, the darkfield attachment is not removed. Darkfield illumination is provided by replacing the standard microscope condenser with a special darkfield condenser which converts light rays to an oblique rather than a direct path pattern. The oblique illumination is reduced by inserting a funnel stop in the lens housing of the oil immersion objective. The light is concentrated by placing immersion oil between the top of the condenser and the bottom of the slide, and between the top of the coverslip and the objective lens. The attachment is adjusted in the following manner:

(1) Remove the eyepiece (ocular) and substage mirror (if present) from the microscope.

(2) Incline the microscope and remove the standard condenser by loosening the set screws.

(3) Insert the darkfield condenser, permitting the lateral adjustment screws to be positioned forward for ease of adjustment.

(4) Place a bright substage lamp (remove the blue filter, if present) directly under the darkfield condenser.

(5) Insert a funnel stop in the housing of the oil-immersion objective.

(6) Set the stage horizontally.

(7) Using low power, sight down the tube (ocular removed) of the microscope and adjust the condenser, by means of the adjustment screws, until a bright ring of illumination appears centered on the surface of the condenser.

(8) Replace the ocular and insert the glass slide containing the material for examination, collected and prepared by the technique described in chapter 3.

(9) Lower the substage condenser, and place a drop of immersion oil on its upper surface.

(10) Raise the substage until the oil contacts the bottom of the slide.

(11) Adjust the condenser and focus to obtain a field in which objects appear bright against a black background.

(12) A preparation of a small amount of blood suspended in saline gives an excellent "focal substance" for the initial adjustment.

b. **Treponema pallidum.** Diagnosis of syphilis rests primarily upon clinical findings, demonstration of spirochetes in chancre material, and serological tests of patient's serum. The serous exudate from a chancre is examined by the method of darkfield illumination for typical slender spirals, measuring 5 to 15 μm in length and 0.2 μm in breadth. Silver or gold impregnation techniques may be used to stain the organisms in tissue. Artificial media, embryonated eggs, and tissue culture have thus far failed to support the organism. Chancre-like penile lesions are not always caused by *Treponema* but are often caused by bacteria, very likely *Neisseria gonorrhea*. Therefore, gram stains should be performed routinely on purulent exudates from the genital area. Staining the *Treponema* by the Gram's technique is usually unsuccessful. Although the organism may stain to a small degree, the staining is not sufficient to render it visible. As previously mentioned, staining can be satisfactorily accomplished by a silver impregnation technique. Two such techniques are the Fontana-Tribondeau method and the Levaditi method. The Fontana-Tribondeau method is based on the coating of the treponeme with reduced silver nitrate, while the Levaditi method coats it with metallic silver. In the absence of silver impregnation some success may be obtained with India ink or nigrosin preparations. This method does not stain; it instead provides a dark background around the organisms making it visible by contrast. Fluorescent antibody

techniques are in widespread use in the detection and confirmation of the presence of treponemes.

c. **Borrelia spp**. The demonstration of spirochetes from patients with recurrent fever is best achieved by collecting blood specimens during the febrile period and examining these specimens immediately by darkfield illumination. Direct smears from peripheral blood may be made during the febrile period; these smears are usually stained with Giemsa's stain. The organisms in dried blood smears stain with common laboratory stains but show much distortion in size and spirals. The organisms grow readily in inoculated rodents but not on artificial media. *Borrelia* (*Treponema*) *vincentii* organisms may be stained by Loeffer's methylene blue, Gram's crystal violet, carbol fuchsin, or Giemsa's or Wright's stain. Smears from the deeper parts of ulcers often show the spirochetes.

d. **Leptospira interrogans (icterohemorrhagiae)**. The darkfield examination is best utilized on blood specimens collected during the early febrile stages or from urine collected on approximately the fifth day after onset of the disease. Infections with *Leptospira* spp are best demonstrated by inoculation of the blood sample to Fletcher's semisolid medium or Schuffner's modified medium. For the precise technique of blood culture, refer to chapter 2. Animal inoculation tests may be accomplished by the intraperitoneal injection of 0.5 ml of urine into fully mature guinea pigs or 21-day old hamsters. Obtain blood specimens from the inoculated animals. At the end of the second week following inoculation, sacrifice the animals and culture the blood and macerated kidney tissue. Agglutination tests are not routinely performed.

Chapter 12
SENSITIVITIES

12–1. Introduction. The determination of antibiotic sensitivity of a particular bacteria is equally as important to the physician as is the identity of the organism. Knowing the organism is an integral part of the treatment because the course of the disease can be predicted and treatment better managed on the basis of the organism's past behavior in infection. However, there are times when the antibiotic sensitivity may be considered more important, and the report of the organism's identity may be delayed. The determination of antibiotic sensitivity of the bacteria is important to the physician in selecting the initial therapeutic course for a patient. If the patient does not respond to the treatment and a change in the antibiotic prescribed must be made, sensitivity studies again become an important tool in patient care. Determinations must be reliable as to results reported. Results must be reproducible with good clinical correlation. Also, if a patient is transferred to another facility, the initial determinations should correlate with those performed at the new laboratory. To achieve this end, several standard methods of sensitivity testing are discussed. These are the paper disc method, the tube dilution method, and the Kirby-Bauer method.

12–2. Paper Disc Method (Basic Sensitivity Method). In this method, a paper disc containing a predetermined concentration of antibiotic is placed on the surface of an agar plate previously inoculated with the organism to be tested (see figure 12–1). The method takes advantage of the fact that the antibiotic in the disc diffuses outward to give a clearly recognizable circular zone of inhibition of the bacteria growing on the medium.

a. **Technique:**

(1) After the organism has been isolated, suspend the suspected pathogen in 0.5 ml of suitable broth.

(2) Incubate the broth 2 to 4 hours at 37°C.

(3) Inoculate the surface of the agar plate(s) as uniformly as possible with the broth culture using a cotton swab.

(4) Allow the surface to dry (covered) for about 10 minutes at room temperature.

(5) Place the sensitivity disc on the inoculated surface with a disc dispenser, a flame sterilized forcep, or a sterile hypodermic needle, making sure there is good contact between the disc and the medium. This can be accomplished by gently pressing the disc in place with a flame-sterilized forceps or an applicator stick.

(6) Incubate the plate(s) for 12 to 18 hours at 37°C and then observe for a zone of inhibited growth surrounding each disc.

b. **Interpretation and Comments:**

(1) *High Potency Disc Method.* Frequently, only one concentration is used for a screening test (the high potency disc). If only one concentration is used for each antibiotic, report the presence or absence of a zone of inhibited growth surrounding each disc.

(2) *Method Using Graded Concentrations.* In order to determine a semiquantitative relationship between concentrations, this test uses two or three graded concentrations for each antibiotic tested. This allows for a comparison of varying concentrations of the same antibiotic, as well as a comparison between different antibiotics. Basically, the units of concentration are expressed in micrograms (μg), or International Units (IU) per milliliter. Table 12–1 shows the procedures for preparation of stock solutions of varying concentrations. It is important that the technician utilize the interpretation sheets or charts which the manufacturer's sheets are generally based on Kirby-Bauer's interpretation of bacterial zones of inhibition. The sensitivity of an organism to a particular antibiotic should not be determined by the presence of a zone surrounding the disc. The size of a zone surrounding a disc is dependent upon such factors as the amount of inoculum, depth of the medium, and also the diffusion and solubility of the antibiotics. The detection of isolated colonies growing within a zone of inhibition indicates the presence of resistant organisms from the culture.

12–3. The Tube Dilution Method. This method employs the serial dilution technique: decreasing concentrations of antibiotics are prepared in broth for inoculation with a culture of the organism to be tested. After inoculation and incubation of the dilutions with the organism, its sensitivity is then determined by the presence or absence of growth in the varying concentrations of therapeutic agents. These determinations can be made in most laboratories, using routine serological tubes with the desired

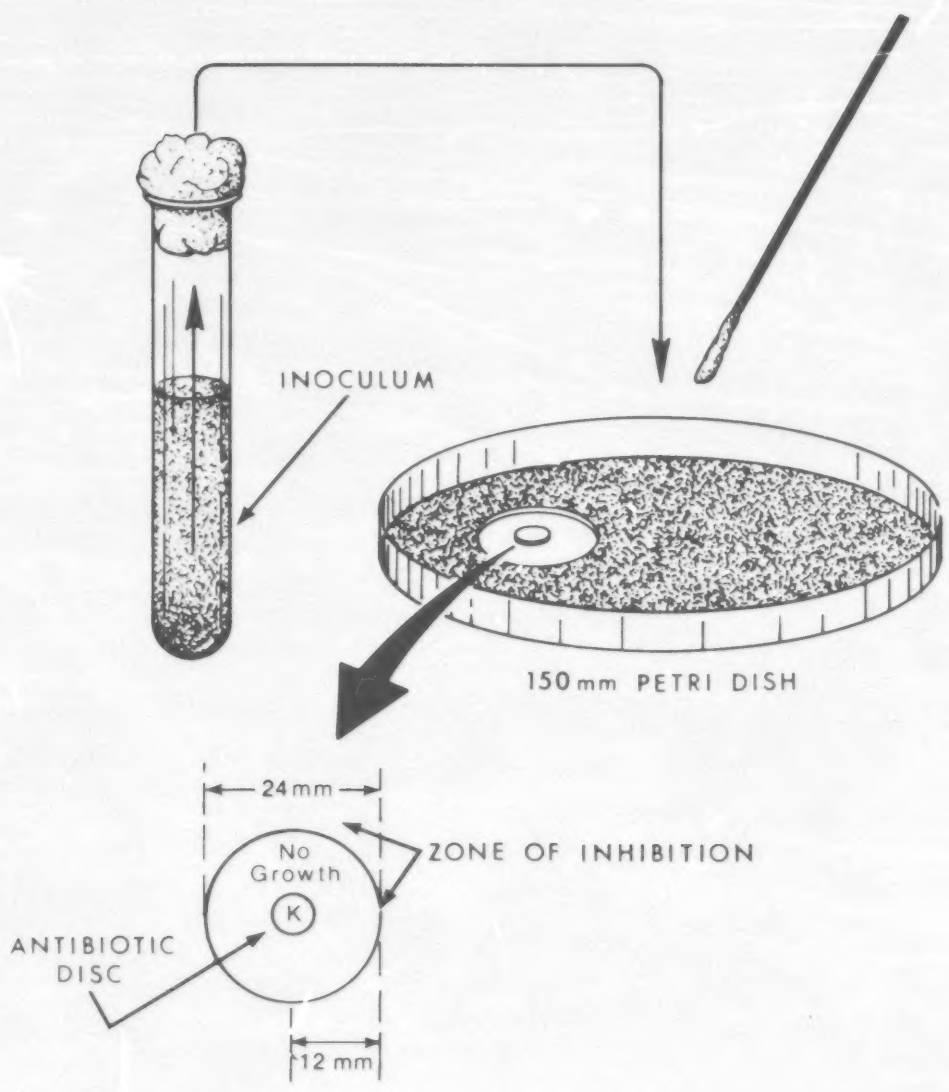


Figure 12–1. Illustration of Kirby-Bauer Technique (Single Disc Used).

antibiotic and an appropriate broth medium. Table 12–2 shows the tube dilution method of sensitivity determination.

a. **Technique:**

(1) Select 18 sterile serological tubes labeling them consecutively from 1 through 18.

(2) Pipette, aseptically, 3.6 ml of sterile trypticase soy or other appropriate broth into tube #1.

(3) Pipette, aseptically, 2.0 ml of the broth into the remaining tubes, labeling tube #18 as the "control."

(4) Prepare a stock antibiotic solution of conventional concentration (generally 1,000 mμg per ml) by the method given in table 12–1. Example: Aqueous penicillin is available in 1,000,000 units per 10 ml.

(5) Prepare a stock solution by withdrawing 1 ml (100,000 mμg) from the vial and diluting to a concentration of 100 mμg/ml.

(6) Pipette, aseptically, 0.4 ml of the stock solution of antibiotic to tube #1. The resulting dilution of antibiotic in tube #1 will now be 100 mμg/ml (see table 12–2). Additional information on dilutions for other common antibiotics are listed in table 12–3.

(7) Using a sterile pipette, mix the contents of tube #1 and transfer 2 ml of solution

Table 12-1. Preparation of Stock Solution of Antibotic.

Quantity of antibiotic		*Sterile water for dilution*	*Resulting concentration of stock solution*
gm	*mg*	*ml*	*µg per ml*
0.1	100	100	1000
0.25	250	250	1000
0.5	500	500	1000
1.0	1000	1000	1000

NOTE: 1 milligram (mg) = 1000 micrograms (µg). The following concentrations are recommended for stock solutions of the more common antibiotics: (1) penicillin 100 units per ml, (2) streptomycin, aureomycin, chloromycetin, and terramycin 1000 µg per ml.

from tube #1 to tube #2, continuing in the same manner to tube #17.

(8) Tube #18 receives no antibiotic and serves as the "control."

(9) Next, inoculate each tube with one drop of a broth culture of the organism to be tested.

(10) Incubate all tubes at 37°C for 12 to 18 hours.

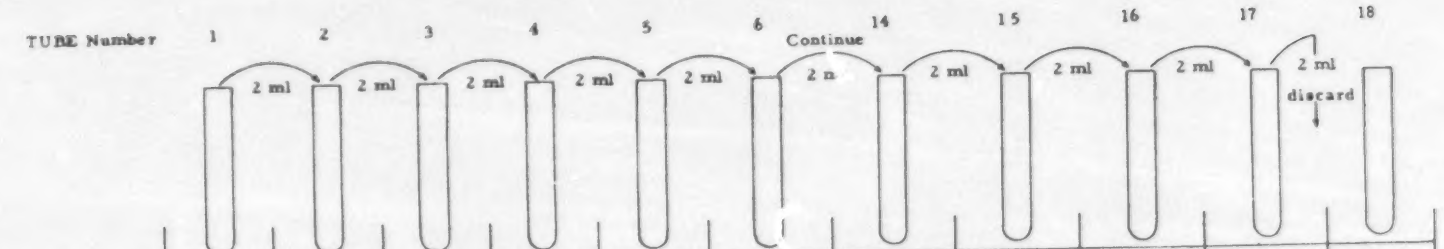
(11) At the end of the incubation period, inspect all tubes for the presence of growth.

b. **Interpretation and Comments.** If there is no growth in the control tube, the test should be considered invalid. If there is good growth in the control tube, look for the tube containing the lowest concentration of antibiotic in which growth is inhibited. If there is a clear-cut distinction between two adjacent tubes of increasing antibiotic concentration, report the result as the lowest concentration of antibiotic in which bacterial growth is inhibited. If there is a gradual decrease in the amount of growth as the concentration of antibiotic increases, report the result as the lowest concentration of antibiotic to which the bacterial growth is sensitive and the lowest concentration of antibiotic to which the growth is completely resistant. The tube dilution method is applicable to research procedures and other situations requiring quantitative determinations, such as SBE and chronic nephritis. Determining the sensitivity of bacteria to sulfa drugs or other synthetic antimicrobial agents can be accomplished using the fundamentals of the techniques for testing sensitivity of bacteria to antibiotics.

12-4. Kirby-Bauer Sensitivity Method. The Kirby-Bauer procedure is an improved method of performing in vitro sensitivity studies. It provides a better picture in regard to a patient's treatment and response when antibiotic therapy is being utilized. It also standardizes sensitivity studies to the point that they correlate to a significant degree between laboratories which use this procedure.

a. **Principle.** The Kirby-Bauer method is based on correlations made between zone sizes (inhibition zones), using quantitative dilutions of antibiotics and corresponding blood levels (circulating antibiotics) in cases of antibiotic therapy. The technique requires use of a standard size petri dish, standardized suspensions of inoculum, and an incubation period of 12 to 18 hours, which results in regular zone measurement (refer again to figure 12-1). Zone measurement is accomplished by use of a millimeter rule and measurement from the center of the zone of inhibition to the outer edge of the zone, then multiplying the result by 2. (Ex-

Table 12-2. Preparation of the Tube Dilution Method (Antibiotic Sensitivity).



Bacterial Suspension, ml (steps 9 - 11)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Antibiotic Being Tested, ml (steps 4-8)	0.4										Control
Add broth, ml (steps 1-3)	3.6	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Resulting Conc. Antibiotic (mcg per ml)	100	50	25	12.5	6.25	(6) 3.1 (7) 1.5 (8) 0.75 (9) 0.37 (10) 0.185	(11) 0.092 (12) 0.045 (13) 0.027 (14) 0.013	0.006	0.003	0.0017	Control

Table 12-3. Procedures for Preparing Stock Solutions.

ANTIBIOTIC	MANUFACTURER	METHOD OF PREPARATION
Ampicillin	Bristol-Myers Co.	Weigh out material and multiply by "activity standard" provided by manufacturer.* Add 0.1 ml. of pH 8.0 phosphate buffer to dissolve; dilute with pH 6.0 phosphate buffer.
Penicillin G	Eli Lilly & Co.	Add 60 ml. of water to a vial containing 1 million units or 600,000 mg; this makes a stock solution of 10,000 mg/ml.
Methicillin	Bristol-Myers Co.	Weight out material and multiply by "activity standard" from manufacturer*; dilute with pH 6.0 phosphate buffer.
Oxacillin	Bristol-Myers Co.	Add 16 ml. of water to a vial to give 1,000 µg/ml.
Cephalothin	Eli Lilly & Co.	Weight out material and multiply by the "activity standard," from the manufacturer*; dilute with pH 6.0 phosphate buffer.
Cephaloridine	Eli Lilly & Co.	Weigh out exactly 30 mg, add 30 ml. of pH 6.0 phosphate buffer to give 1,000 µg/ml.
Carbenicillin	Beecham-Massengill Pharmaceuticals	Add 10 ml. of water to a vial containing 1 gm. of drug, then dilute 1:100 to give 1,000 µg/ml.
Tetracycline	Pfizer Lab.	Add 20 ml. of water to a vial that contains 20 mg of drug, to give 1,000 µg/ml.
Chloramphenicol	Parke, Davis & Co.	Weight out exactly 50 mg, add 1 ml. of ethyl alcohol to dissolve drug and sufficient water to 50 ml. This gives a concentration of 1,000 µg/ml.
Erythromycin	Abbott Laboratories	Weigh out material and multiply by the "activity standard" from the manufacturer.* Dissolve in 1-2 ml. alcohol and add water to a final concentration of 1,000 µg/ml.
Lincomycin	The Upjohn Co.	Add 20 ml. water to a vial to make 1,000 µg/ml.
Clindamycin	The Upjohn Co.	Add 15 ml. of water to vial containing 150 mg of drug, dilute 1:10 to give 1,000 µg/ml.
Kanamycin	Bristol-Myers Co.	Weigh out exactly 30 mg, add 30 ml. water to give 1,000 µg/ml. Agent is unstable in acid range.
Gentamicin	Schering Corp.	Weigh out material and multiply by "activity standard" from manufacturer,* dilute with water to give 1,000 1,000 µg/ml.
Polymyxin B	Burroughs Wellcome & Co.	Add 5 ml. water to a vial containing 50 µg, to give 10,000 µg/ml.; dilute to desired concentration.
Colistin (polymyxin E)	Warner-Chilcott Lab.	Weigh out material and multiply by "activity standard" from manufacturer,* dilute with water to desired concentration.
Bacitracin	The Upjohn Co.	Add 10 ml. of water to a vial containing 10,000 units, to give 1,000 units/ml.
Nitrofurantoin	Eaton Labs., Inc.	Weigh out 120 mg of drug and transfer to a 50-ml. flask containing 4.0 ml. of dimethyl formamide. Heat in 56°C. water bath with shaking to dissolve. This solution contains 30 mg/ml.
Nalidixic acid	Winthrop Labs.	Weigh out approx. 50 mg, add 2 ml. of 1 N NaOH and allow to stand to dissolve (may require gentle heat). Dilute with sterile water (less 2 ml.) to desired concentration.

*Courtesy of John A. Washington II, Head, Section of Clinical Microbiology, Mayo Clinic.
*Example: 1 mg = 825 µg ("activity standard"/µg). 50 mg = 50 x 825 = 41,250 µg. Therefore, add 41.2 ml. of diluent to give 1,000 µg/ml.

ample: If a zone measures 12 mm from the center to the edge of bacterial growth, the zone of inhibition is 2 # 12 × 24. This means the diameter of the zone of inhibition is 24 mm with respect to the circle.) Based on these measurements, zones of inhibition can be categorized as resistant, immediate, or sensitive, depending on the antibiotic concentration of the disc used in the test (see figure 12-2).

b. **Technique.** The Kirby-Bauer sensitivity procedure is also known as a high-potency disc method. The term "high-potency" arises from the earlier use of both low and high concentration discs in the performance of sensitivity studies. The use of a single high potency disc has replaced this practice which required several discs containing varying strengths of antibiotics. The procedure is performed using Mueller-Hinton agar as the basic sensitivity medium:

(1) Prepare Mueller-Hinton agar, using the instructions on the label of the jar; sterilize the medium and allow it to cool.

(2) Distribute 75 to 80 ml of agar into 150-mm plastic plates and allow them to solidify. (These larger plates are used in give

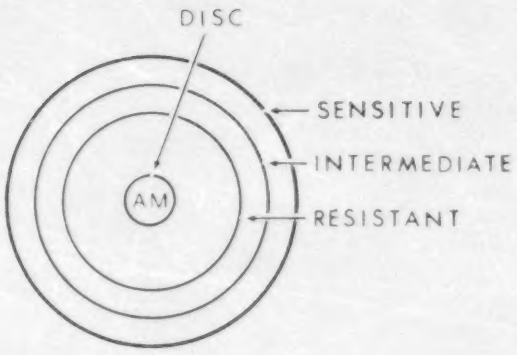


Figure 12–2. Illustration of Inhibition Zones Using Ampicillin (10 ug).

ample room for zones of antibiotic diffusion through the media.) Use the plates within 5 days for best results.

(3) Prepare the bacteria to be tested by placing small samples of 5 to 10 colonies in 4 ml of trypticase soy broth, incubating until the organisms are just visible when the tube is subjected to transmitted light. The standardized inoculum suspension should visually match a barium sulfate turbidity standard prepared as follows: Add 0.5 ml of a 1 percent barium chloride (11.8 g l $BaCl=$, $2H_2O$) solution to 99.5 ml of 1 percent sulfuric acid. If the tube is swirled gently, the media will have a silky look against the lighted background.

(4) Swab the standardized inoculum described below evenly over the agar surface and allow it to absorb for about 10 minutes prior to application of the disc.

(5) Incubate inoculated plates for 18 to 24 hours prior to observation for zones of inhibition. Replace the turbidity standard once each month because particles may become larger over a longer period of time. Make sure that the sensitivity discs are not outdated and that they have not been stored improperly, to protect disc potency. Never use outdated discs because potency may be affected by aging. The incubation of plates overnight is usually sufficient to obtain growth for interpretation of the sensitivity. Some organisms may require a shorter or a longer incubation period.

(6) Where the use of 150-mm plates is not practical due to cost and the number of tests performed, the method can be modified by simply using standardized inoculum, Mueller-Hinton agar, and regular size plates. If smaller plates (routine size) are used, fewer discs should be placed on the inoculum in order to allow for diffusion of the antibiotic from the disc. Such a procedure will require a number of plates to accomplish a single sensitivity battery. In figure 12–3, it can be seen that the smaller plate is crowded and disc numbers 1 and 7 and numbers 3 and 9 are overlapping in their zones of inhibition. This can cause a misreading of diffusion points and lead to an incorrect reading of the size of the zone of inhibition. In contrast, the larger plate can be seeded with 12 discs, and there will be no overlapping of inhibited zones.

c. **Interpretation:**

(1) Resistance to an antibiotic is indicated when the zone of inhibition is very small surrounding the sensitivity disc (see figure 12–2).

(2) A sensitive reaction is one in which the zone of inhibition clearly indicates that the antibiotic has stopped bacterial growth at the maximum point of antibiotic diffusion.

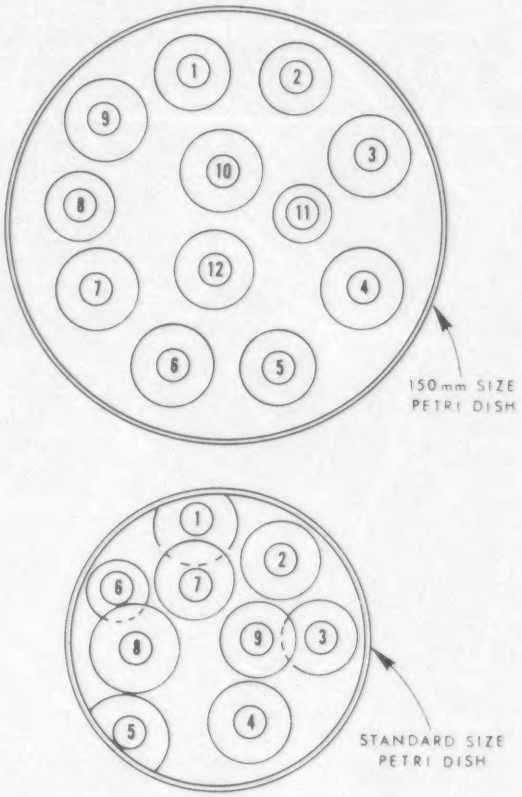


Figure 12–3. Comparison of 150–mm and Standard Size Petri Dish (Kirby-Bauer Sensitivity).

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Table 12-4. Schema for Recommended Antimicrobial Discs.

Isolate	AM 10 µg	CB 50 µg	CF 30 µg	CP 30 µg	CT 5 µg	CR 15 µg	CM 30 µg	KN 30 µg	LN 2 µg	OT 1.2 µg	SA 10 µg	SF 100 µg	TE 30 µg	VB 100 U	PN 10 U	TC 30 µg
[illegible]																
[illegible]	x	[x]	x	[x]			x	x			[x]	[x]		x	[x]	[x]
Citrobacter-Enterobacter-Klebsiella	x		x	[x]			x	x			[x]	[x]		x	[x]	[x]
[illegible]	x		x	[x]			x	x			[x]	[x]		x	[x]	[x]
[illegible]	x	[x]		[x]			x	x			[x]	[x]			[x]	[x]
[illegible]		x		[x]			x				[x]			x	[x]	[x]
[illegible]	x	x		[x]			x	x			[x]	[x]		x	[x]	[x]
[illegible]																
[illegible]			x	[x]	x	x	[x]		[x]	x			x			[x]
[illegible]						[x]			[x]				[x]			[x]
Group D streptococci, including enterococci	x		x	[x]		x	[x]						x		[x]	[x]
[illegible]			[x]						[x]				[x]			[x]
Other streptococci (alpha, and so forth)	x		x	[x]		x							x			[x]
Miscellaneous gram+																
Staphylococcus aureus/epidermidis	x		x	[x]									x			[x]
[illegible]	x		x	[x]									x		[x]	[x]
Other facultative organisms	As indicated by identity of isolate															

*From the Section of Microbiology and the Infectious Disease Research Laboratory, Wilmington Medical Center, Wilmington, Del. Abbreviations: AM, ampicillin; CB, carbenicillin; CF, cephalothin; CM, chloramphenicol; CL, clindamycin; EM, erythromycin; GM, gentamicin; KN, kanamycin; LN, lincomycin; P, methicillin; SA, nalidixic acid; SF, nitrofurantoin; PN, penicillin G; PX, polymyxin B; SM, streptomycin; TE, tetracycline. x, Recommended for use with the species indicated; [x], recommended for use with urine isolates only; [x], recommended for use with blood culture isolates only.

(3) An intermediate reaction is one in which the zone measurement indications are neither resistant nor sensitive.

(4) Antibiotic diffusion is the radial spread of the antibiotic through the medium.

(5) Information on how to interpret the results of a sensitivity test can be found on the instructions received with the particular disc used, along with schema provided in table 12-4 as a practical guide for disc selections.

(6) A summary of the zone size interpretations is shown in table 12-5.

Table 12–5. Zone Size Interpretative Chart, Kirby-Bauer Method.

ANTIBIOTIC OR CHEMOTHERAPEUTIC AGENT	DISC POTENCY	INHIBITION ZONE DIAMETER TO NEAREST MM. RESISTANT	INTERMEDIATE	SENSITIVE
Ampicillin[1]				
Enterobacteriaceae and enterococci	10 µg.	11 or less	12–13	14 or more
Staphylococci		20 or less	21–28	29 or more
Other organisms		11 or less	12–21	22 or more
Bacitracin	10 U.	8 or less	9–12	13 or more
Carbenicillin	100 µg.			
Pseudomonas sp.		13 or less	14–16	17 or more
Proteus and *Escherichia coli*		17 or less	18–22	23 or more
Cephaloridine	30 µg.	11 or less	12–15	16 or more
Cephalothin	30 µg.	14 or less	15–17	18 or more
Chloramphenicol	30 µg.	12 or less	13–17	18 or more
Clindamycin[2]	2 µg.	11 or less	12–15	16 or more
Colistin (polymyxin E)[3]	10 µg.	8 or less	9–10	11 or more
Erythromycin	15 µg.	13 or less	14–17	18 or more
Gentamicin	10 µg.	12 or less	13–14	15 or more
Kanamycin	30 µg.	13 or less	14–17	18 or more
Lincomycin	2 µg.	9 or less	10–14	15 or more
Methicillin[4]	5 µg.	9 or less	10–13	14 or more
Nafcillin and oxacillin	1 µg.	10 or less	11–12	13 or more
Nalidixic acid[5]	30 µg.	13 or less	14–18	19 or more
Neomycin	30 µg.	12 or less	13–16	17 or more
Nitrofurantoin[5]	300 µg.	14 or less	15–18	19 or more
Penicillin G				
Staphylococci	10 U.	20 or les	21–28	29 or more
Other organisms	10 U.	11 or less	12–21[6]	22 or more
Polymyxin B[3]	300 U.	8 or less	9–11	12 or more
Rifampin (when testing *Neisseria meningitis* susceptibility only)	5 µg.	24 or less		25 or more
Streptomycin	10 µg.	11 or less	12–14	15 or more
Sulfonamides[5,7]	300 µg.	12 or less	13–16	17 or more
Tetracycline[8]	30 µg.	14 or less	15–18	19 or more
Vancomycin	30 µg.	9 or less	10–11	12 or more

*Courtesy Alfred W. Bauer, Group Medical Center, Seattle, Wash., and John C. Sherris and W. Lawrence Drew, University Hospital, Seattle, Wash. See also reference 3 at end of chapter. Updated and modified by other investigators. See references 9 and 14.

[1]The ampicillin disc is used for testing susceptibility to both ampicillin and betacillin.

[2]The clindamycin disc is used for testing susceptibility to both clindamycin and lincomycin.

[3]The polymyxins diffuse poorly in agar, and the accuracy of the diffusion method is less than with other antibiotics. Resistance is always significant, but some relatively resistant strains of *Klebsiella* and *Enterobacter* may give zones in the lower end of the sensitive range (up to 15 mm.). When treatment of systemic infections due to susceptible strains is considered, it is wise to confirm the results of a diffusion test with a dilution method.

[4]The methicillin disc is used for testing susceptibility to all penicillin-resistant penicillins: methicillin, cloxacillin, dicloxacillin, oxacillin, and nafcillin. Methicillin-resistant strains of *Staphylococcus aureus* are best detected at 30°C.

[5]Urinary tract infections only

[6]This category includes some organisms, such as enterococci and gram-negative bacilli, that may cause systemic infections treatable by high doses of penicillin G.

[7]Any of the commercially available 300 or 250 µg. sulfonamide discs can be used with the same standards of zone interpretation.

[8]The tetracycline disc is used for testing susceptibility to all the tetracyclines: chlortetracycline, demeclocycline, doxycycline, methacycline, oxytetracycline, rolitetracycline, minocycline, and tetracycline.

Chapter 13
BACTERIOLOGY OF FOOD

13–1. Introduction. Food bacteriology in this manual is concerned with the cultivation, isolation, and identification of those bacteriological agents responsible for outbreak of disease following the ingestion of contaminated foods. The investigation of contamination in food may be required at some time. In such cases, consult AFM 163–8, chapter 5, paragraph 5–1, for the nonlaboratory aspects of food poisoning outbreaks.

13–2. Collecting and Processing Specimens. Suspicious food specimens should be collected promptly, and the laboratory examination should be started without delay. Convenient containers to use for the collection of food specimens are sterile containers with tight-fitting lids.

a. The techniques used for the bacteriological examination of food are essentially the same as those used for the bacteriological examination of water and dairy products. These techniques include the direct microscopic examination of liquid mounts or stained portions of food; plate counts to determine the total number of viable bacteria; selective cultivation, including coliform counts to determine the presence of bacterial types which may be indicative of improper handling or processing; and tests for specific pathogens.

b. The genera most frequently implicated in food poisoning include *Staphylococcus*, *Streptococcus*, *Salmonella*, and *Clostridium*. Procedures for the detection of the genus Clostridium are taken up as a special entity at the end of this chapter. Routine food analysis will be presented first.

13–3. Routine Food Analysis. The routine procedure for the analysis of food is (1) direct examination by microscope, (2) primary isolation, and (3) the examination of cultures.

a. **Direct Microscopic Examination:**

(1) Prepare a direct smear of solid or liquid food by placing or smearing a small amount of the material on a glass slide.

(2) Fix the material by first air-drying and then heat-fixing over a flame, taking care not to overheat the material.

(3) Gram-stain the smear.

(4) Examine the smear microscopically for the predominating morphological types.

b. **Primary Isolation:**

(1) Place an inoculating loop of suspected materials on two blood agar plates and two mannitol salt agar plates.

(2) Streak one differential agar plate, such as MacConkey, casein-methylene blue, or desoxycholate agar; then streak two agar plates of the more selective media, such as Salmonella-Shigella or desoxycholate-citrate agars.

(3) For the detection of *Salmonella* spp, bismuth sulfite agar and brilliant green agar should be inoculated.

(4) Inoculate a 25- or 50-ml aliquot of filtered suspension of the food sample under investigation into an equal volume of selenite F or tetrathionate broth.

(5) Incubate plates and broth for 24 hours at 37°C for 18 to 22 hours.

(6) A quantitative plate may be prepared by diluting the sample to 1:100, 1:1000, or 1:10000, using sterile buffered water beginning with the routine dilution of 1 ml of the sample to 99 ml of the diluent.

c. **Examination of Cultures:**

(1) Examine the streaked agar plates for species of Staphylococcus or Streptococcus.

(2) Perform the coagulase test on colonies of staphylococci producing a clear zone on DNase agar following the addition of 1 NHC1. (See paragraph 5–6a.)

(3) Streptococcal strains should be inoculated to SF medium or any other appropriate medium for differentiation of *Streptococcus faecalis* from other streps. Consult chapter 5 on the final identification.

(4) Count the predominating organisms that are present in the medium used to quantitate the determination.

(5) If suspicious colonies are found on the differential and selective plates, proceed with identification of suspected intestinal pathogens according to directions in chapter 8.

13–4. Examination of Foods for *Clostridium botulinum*. The demonstration of *Clostridium botulinum* toxin in leftover food or drink is essential in confirming a diagnosis of botulism. This may be accomplished by inoculating extracts of suspected materials into laboratory animals. *C. botulinum* may be cultivated from the remaining food and the resultant pure culture similarly tested for evidence of toxin production. Although animal inoculation is not

a routine procedure in most military hospitals, the procedure is included here for the benefit of those facilities capable of performing the test. Small facilities will simply collect samples and submit them to a reference service or facility.

a. **Culture Specimens:**

(1) The food materials submitted may be canned goods, smoked meat, or spiced meats. Such materials may be obviously spoiled and rancid, or they may appear harmless. The liquid from canned foods may be examined for toxin.

(2) If dry or solid foods are to be examined, infusions should be prepared in a minimal amount of physiological saline.

(3) With meat samples, meat infusions are best prepared by macerating portions of the specimen before adding sterile normal saline. The mortar and pestle for the preparation must be sterile.

b. **Isolation and Identification:**

(1) Inoculate each of three tubes of cooked meat medium or thioglycollate with approximately 1 g or 1 ml of food sample. The medium should contain 0.1 percent dextrose to facilitate growth of this anaerobe. The final pH should be about 7.1 after autoclaving.

(2) Inoculate two blood agar plates with the specimen, incubating the plates anaerobically in a candle jar for 48 to 72 hours.

(3) In examining the blood plates, look for typical growth and prepare a gram stain of the organism. A spore stain may be prepared at this point, if desired.

(4) Stain growth from the cooked meat medium and examine for the typical morphology.

(5) Final identification of Clostridia should be confirmed by procedures given in chapter 7.

c. **Preparation for Virulence Testing:**

(1) Transfer the liquid (infusion) or saline suspension of the food to a sterile screw-capped tube using a sterile rubber-bulbed pipet.

(2) Centrifuge this mixture at 4500 rpm for 1 hour.

(3) After centrifugation is completed, transfer equal volumes of the supernate into each of two sterile, screw-capped tubes. (Label these tubes #1 and #2.)

(4) Heat the tube marked #2 in a boiling water bath for 10 minutes. This tube will serve as a heat-labile toxin control.

d. **Animal Inoculation:**

(1) Select 8 white mice or guinea pigs for inoculation and controls; number them 1 through 8, using a laboratory marker (pen, tag, paint, etc).

(2) Make all animal injections intraperitoneally. If mice are used, inject 0.5 ml of the supernate. If guinea pigs are used, inject 1.0 ml. Refer to table 13–1.

(3) Inject animals 1 and 2 with the appropriate volume of material from tube #2.

(4) Inject animals 3 and 4 with the appropriate amount of material from tube #1.

(5) Inject animals 5 and 6 with 1.0 ml each of botulinum antitoxin, type A. Then inject each animal with the appropriate volume of material from tube #1.

(6) Inject animals 7 and 8 with 1.0 ml each of botulinum antitoxin, type B. Then inject each animal with the appropriate volume of material from tube 1.

e. **Observation of Test Animals:**

Table 13–1. Inoculation of Animals in Virulence Tests for *Clostridium botulinum*.

Mouse #	*Tube #1 unheated*	*Tube #2 heated*	*Botulinum antitoxin Type A*	*Botulinum antitoxin Type B*
1 & 2	----	0.5 ml	----	----
3 & 4	0.5 ml	----	----	----
5 & 6	(2) 0.5	----	(1) 1.0 ml	----
7 & 8	(2) 0.5	----	----	(1) 1.0 ml

(1) If animals 1, 2, 5, and 6 survive, type A botulinum toxin is present in the specimen.

(2) If animals 3, 4, 7, and 8 survive, type B botulinum toxin is present.

(3) In instances where only animals 1 and 2 survive, further tests should be performed on animals immunized with antitoxin against type E toxin.

(4) If all the animals die, lethal substances are present in the inoculum whether or not heat-labile botulinum toxin is present.

f. **Discussion.** *C. botulinum* toxins A, B, and occasionally E are usually implicated in food poisoning. Virulence tests with C and D antitoxins should be performed when the toxin is not identified. Evidence of toxicity is first noted in animals by the development of flaccid paralysis, rapidly followed by death.

Chapter 14
SPECIAL TESTS

14–1. Introduction. Since special tests are of general applicability throughout this manual, it is deemed appropriate to group them together in a separate chapter. Biochemical tests are cultural and related methods that investigate bacterial nutrition and respiratory processes. Results of these tests provide the primary means of separating bacteria into species, genera, and families, within the taxonomic classification system. These tests are based on the fact that distinctive groups of bacteria utilize limited numbers of nutritive substances and produce metabolic byproducts which are characteristic of their specific groups. The tests discussed in this chapter do not cover all such methods available to the bacteriologist. Only the more common special tests routinely performed are described. Where appropriate, culture medium recipes are given primarily for information purposes. When they are available, the media discussed in this manual should be purchased from a commercial source, such as Difco, BBL, or Fisher, unless otherwise indicated.

14–2. Acid-Fast Bacilli Catalase Test:

a. **Principle.** Acid-fast bacilli produce the enzyme catalase. Catalast activity will be decreased when the bacilli become resistant to the therapeutic agent, isoniazid. The loss of catalase activity also is correlated with a weakening of virulence for the guinea pig. It is possible to subgroup acid-fast bacilli on the basis of their catalase activity at different temperatures and pH.

b. **Room Temperature Method:**

(1) *Reagents:*

(a) Hydrogen peroxide, 30 percent.

(b) Tween 80, 10 percent.

(c) Phosphate buffer solution, pH 7.0.

(2) Procedure:

(a) Prepare a 1:1 mixture of 10-percent Tween 80 and 30 percent hydrogen peroxide.

(b) At room temperature, add 0.5 ml of the Step 1 mixture to a slant containing *Mycobacterium* growth.

(c) Observe for bubbling.

c. **Temperature and pH Effects Method:**

(1) *Reagents.* Same as (1) above.

(2) *Procedure:*

(a) Add several loopfuls of *Mycobacterium* growth from a slant to 0.5 ml of phosphate buffer solution in a test tube.

(b) Incubate in a 68°C water bath for 20 to 30 minutes.

(c) Prepare a 1:1 mixture of 10 percent Tween 80 and 30 percent hydrogen peroxide.

(d) after incubation, add 0.5 ml of the Step c mixture to the buffer growth mixture.

d. **Interpretation.** At room temperature most acid-fast bacilli are catalase positive. Those strains not exhibiting catalast activity are usually isoniazid-resistant, possessing little virulence for guinea pigs. At 68°C and pH 7.0, the catalase activity of human and bovine tubercle bacilli is selectively inactivated. Under the same conditions, all other acid-fast bacilli are catalase positive.

14–3. Acid-Fast Bacilli Concentration Techniques:

a. **Principle.** Specimens of sputum, gastric washings, or other body fluids that contain too few acid-fast bacilli to be found by direct smear may be concentrated by digesting the mucus with sodium hydroxide or trisodium phosphate and then centrifuging.

b. **Sodium Hydroxide Method:**

(1) *Reagents:*

(a) Sodium hydroxide, 4 percent.

(b) Bromthymol blue indicator, 0.2 percent.

(c) Hydrochloric acid, 5 percent.

(2) *Technique:*

(a) Dilute specimen with an equal volume of 4 percent NaOH.

(b) Shake for 10 minutes in mechanical shaker or incubate for 30 minutes at 37°C.

(c) Neutralize with 5 percent HCl using bromthymol blue as an indicator (end point is grass green color).

(d) Centrifuge at 3,000 rpm for 30 minutes.

(e) Decant the supernate into a disinfectant solution.

(f) Use sediment to culture, to make concentrated smears, and to inoculate guinea pigs.

c. **Trisodium Phosphate Method:**

(1) *Reagent:* Trisodium phosphate (TSP) 23 percent.

(2) *Technique:*

(a) Dilute specimen with an equal volume of TSP.

(b) Allow to stand at room tempera-

ture for 24-hours or until digestion is complete.

(c) Centrifuge at 3,000 rpm for 30 minutes.

(d) Decant supernate into a disinfectant solution.

(e) Wash sediment with 8 to 10 ml of sterile saline.

(f) Centrifuge as before, discarding the supernate.

(g) Use sediment to culture, make concentrated smears, or guinea pig inoculation.

d. **Discussion:**

(1) The sodium hydroxide method is ideal for most clinical laboratories but must be neutralized immediately after digestion. Since prolonged exposure to the alkali will eventually kill acid-fast bacilli, some laboratories prefer to neutralize the sediment in lieu of neutralization prior to centrifugation.

(2) Trisodium phosphate is recommended when specimens need not be digested immediately, especially when shipping specimens via mail to reference laboratories. Acid-fast bacilli remain viable for days after exposure to trisodium phosphate.

14–4. Acid-Fast Bacilli Neutral Red Test (Optional):

a. **Principle.** Cells of virulent *Mycobacteria* suspended in barbital buffer solution are bound by the indicator methyl red, while avirulent strains are not affected.

b. **Reagents:**

(1) Methyl alcohol, 50 percent.

(2) Sodium chloride solution, 5 percent.

(3) Sodium barbital.

(4) Aqueous neutral red solution, 0.2 percent.

c. **Procedure:**

(1) Suspend several *Mycobacterium* colonies in 5 ml of 50 percent methyl alcohol.

(2) Incubate for 1 hour at 37°C. Centrifuge and discard the supernate. Repeat the washing procedure using another 5 ml of 50 percent methyl alcohol.

(3) After discarding the supernate, resuspend the sediment in 5 ml of freshly prepared barbital buffer (equal volumes of 5 percent sodium chloride and 1 percent sodium barbital).

(4) Add 1 ml of aqueous neutral red solution to the growth-buffer mixture. Allow to stand for 30 minutes at room temperature before reading.

d. **Interpretation:**

(1) Cells of virulent *Mycobacteria* develop red color.

(2) Avirulent *Mycobacteria* remain yellow in the alkaline barbital solution.

14–5. Acid-Fast Bacilli Niacin Test:

a. **Principle.** *Mycobacterium tuberculosis* produces niacin. Niacin combines with cyanogen bromide and develops a yellow color.

b. **Reagents:**

(1) Sterile water.

(2) Ethyl alcohol, 95 percent.

(3) Cyanogen bromide, aqueous, 10 percent.

(4) Aniline, alcoholic, 4 percent.

c. **Procedure:**

(1) Add 0.5 to 1.0 ml of sterile water to a slant culture of acid-fast bacilli.

(2) Place culture tubes at such an angle as to allow the water to layer over the colonies. This will extract any niacin present within 5 to 10 minutes.

(3) Transfer 0.5 ml of fluid from slant to a test tube. Add equal quantities of 4-percent aniline in ethyl alcohol and 10-percent aqueous cyanogen bromide. CAUTION: AVOID INHALATION OF TOXIC CYANOGEN BROMIDE FUMES.

(4) Gently shake the tube and observe for color change.

d. **Interpretation.** The presence of niacin is evidenced by the immediate development of a yellow color throughout the mixture.

14–6. Agglutination Test:

a. **Principle.** Agglutination will occur when a specific antigen (organism) is mixed with its corresponding antiserum.

b. **Reagent.** Commercially prepared antisera.

c. **Technique:**

(1) Mark a slide, width-wise, with a series of parallel wax pencil lines, approximately ¼-inch apart.

(2) Deliver a drop of the suspended organism near the edge of the slide in each lined area.

(3) Add a drop of appropriately diluted antiserum to the other end of each lined area.

(4) One of the two remaining areas should receive a drop of saline instead of antiserum; the other a drop of normal rabbit serum. These will serve as controls.

(5) Carefully mix together the droplets of each lined area with a loop. Take care to prevent cross-mixing.

(6) Gently rock the slide and observe for agglutination. NOTE: The agglutination test

may be performed by mixing the specific antigen (organism) with an antiserum in a test tube, rather than on a slide.

d. **Interpretation.** The particular antiserum in which agglutination occurs most rapidly and completely is the group or subgroup to which the organism belongs.

14-7. Bile Solubility Test:

a. **Principle.** Pneumococci are lysed in the presence of bile salts; streptococci are not lysed. This test is used to distinguish between the alpha hemolytic streptococci and pneumococci, which are also alpha hemolytic.

b. **Reagents and Media:**

(1) Sodium desoxycholate (bile salt) 2 percent.

(2) Physiological saline.

(3) Phenol-red indicator.

(4) Sodium hydroxide, 0.1 N.

(5) Thioglycollate broth or Todd-Hewitt broth.

c. **Technique:**

(1) Inoculate a tube of thioglycollate broth or a tube of Todd-Hewitt broth with the bacteria in question.

(2) Incubate the broth culture for 18 to 24 hours at 37°C. NOTE: An alternative procedure is to prepare a heavy suspension of the bacteria to be tested in 1.0 ml of physiological saline and proceed to step 3.

(3) Because of the fermentation of glucose, and the resulting lactic acid formation in streptococcal or pneumococcal cultures, adjustment of pH to neutral is necessary before proceeding with the test. Adjust the pH of the broth culture (or saline suspension) by adding one drop of phenol red indicator (or other suitable indicator) to the latter and then titrating with a few drops of 0.1 N sodium hydroxide until a neutral pH is obtained. If phenol red is used, the neutral pH is manifested by a pink color.

(4) Place 0.5 ml of the neutralized broth or saline suspension into each of two *clean* Kahn tubes.

(5) Label one tube "test," and add to this tube 0.5 ml of 2 percent sodium desoxycholate.

(6) Label the second tube "control," and add to this tube 0.5 ml of physiological saline.

(7) Place both tubes in the incubator at 37°C and observe at 1, 2, and 3 hours for clearing in the tube labeled "test" (the tube containing the sodium desoxycholate).

d. **Interpretation:**

(1) If the organism is *(Streptococcus Diplococcus) pneumoniae*, clearing should occur in the tube containing the sodium desoxycholate within the 3-hour incubation period. Alpha streptococci cultures remain turbid like the *control*.

(2) Regard as negative all tubes that do not clear within the 3-hours of incubation.

(3) The cultures *must* be neutralized (step 3) because sodium desoxycholate in an acid solution may form precipitate or gel which interferes with the reading of the test.

14-8. Carbohydrate Utilization Tests:

a. **Principle:**

(1) Sugar, alcohol and other abundant carbohydrates possess a large amount of molecular free energy. Most bacteria are able to convert high energy substances to compounds having less free energy, releasing the excess for use in their life processes. Carbohydrate utilization may occur through aerobic oxidation or through fermentation, an anaerobic process.

(2) In the conversion process specific byproducts are formed. For example, when glucose is utilized by a bacterium the following events occur:

Glucose Intermediate Acids $CO_2 + 2H_2O$

In the conversion of glucose large amounts of detectable acid products are produced followed eventually by the production of significant amounts of carbon dioxide gas and water.

(3) Although most bacteria possess systems for utilizing carbohydrates, all do not. And they differ in the amount of byproduct produced, the time required to produce it, and in other ways. Some species can completely convert a high energy substance to low energy end products; others are capable of converting these substances to intermediate levels, and still others do not possess systems for conversion of these substances.

(4) In carbohydrate utilization tests, bacteria are cultured in a medium containing a single or several specific carbohydrates and they may produce the following:

(a) Acid.

(b) Acid + CO_2.

(c) Not utilize the substance.

(5) Acid production is detected by a dye indicator incorporated in the medium. Several of the most common ones are:

(a) Phenol red: red yellow pH 6.8 to 8.4.

(b) Bromthymol blue: yellow blue pH 6.0 to 7.6.

(c) Bromcresol purple: purple yellow ph 5.2 to 6.8.

(d) Andrade's indicator: colorless red ph 5 to 8.

(6) Carbon dioxide gas (CO_2) is detected as follows:

(a) In solid tubed media: escaping gas disrupts the medium, creating bubbling and tearing of the medium.

(b) In liquid medium: escaping gas is trapped in a Durham gas detection tube previously filled with medium and placed inverted in the tube prior to inoculation. CO_2 is seen as a bubble that displaces the medium in the Durham tube.

(7) Complex analysis of conversion products can be accomplished using gas chromatographic techniques. In these procedures individual intermediate acids and other byproducts are identified and quantitated. This method has enhanced the separation of closely related species.

b. **Media for Carbohydrate Utilization.** Phenol red broth and CTA, supplemented with specific carbohydrates can be used, depending on the characteristics of the organism being tested and whether a liquid or solid media is desired.

c. **Procedures and Interpretation:**

(1) *Phenol Red Broth—Enterobacteriaceae Testing.* Pick a colony from isolation medium and transfer to 0.5 ml of nutrient broth, brain-heart infusion broth, etc. Incubate in 37°C water bath for 30 minutes to an hour. Remove, shake well, and inoculate the tubes of selected carbohydrate media with a small loopful per tube of medium. *Acid* production is indicated when the medium turns yellow. *Gas* is indicated by bubbles in the Durham tube or disruption of the medium if a solid medium is used. Different species of bacteria show different patterns of utilization. Examples of carbohydrate utilization tests are shown in table 8–5.

(2) *Cystine Trypticase Agar (CTA)—Neisseria Testing.* Carefully pick a colony from the isolation medium and transfer it to 0.5 ml of an enriched broth. Incubate in a 37°C water bath or at the appropriate temperature. Shake well and inoculate only the surface of the medium. Other fastidious organisms, such as streptococci, can be inoculated by stabbing the medium to about one-half its depth. *Acid production* is indicated by a yellow color. *Gas* is indicated by bubbling or disruption of the medium. The medium without carbohydrate can be used as a holding medium and for motility detection. Examples of carbohydrate tests in CTA are shown in table 8–5.

d. **Discussion:**

(1) Carbohydrate utilization patterns are an extremely important step in the identification process. Tests must be performed correctly with rigid adherence to temperature, pH, and other limitations.

(2) Tests must be performed in tubes with loose-fitting caps or cotton plugs. If screw-capped tubes are used, the caps must be loosened before the cultures are incubated.

(3) In most media, acid production must be substantial to be detectable. Small amounts of acid produced in peptone utilization are not detected.

(4) Differentiation of oxidative and fermentative acid production is made by utilizing O-F test medium.

(5) *Neisseria* and other fastidious organisms do not produce great amounts of acid; therefore, you should inoculate the surface of the media particularly when testing *Neisseria*. This increases visualization of the indicator color change. Cultures may show an alkaline shift if the carbohydrate is not utilized.

(6) Other basal media recommended by some authorities include purple broth, peptone sugar waters, serum water, and peptone meat-extract broth.

(7) Several satisfactory motility test mediums are available from commercial sources. Some of them, such as SIM, test for several other properties at the same time and are time-saving.

14–9. Catalase Test (Mycobacteria, Staphylococcus):

a. **Principle.** Staphylococci produce the enzyme catalase which, when mixed with hydrogen peroxide, liberates oxygen from the hydrogen peroxide with the occurrence of vigorous bubbling.

b. **Reagent.** Hydrogen peroxide, 30 percent (superoxol). 10 percent Tween 80 1:1 mixture should be added when testing Mycobacteria.

c. **Technique:**

(1) Place a drop of hydrogen peroxide on a clean glass slide.

(2) Pick the colony (colonies) in question from an agar plate by means of a wire loop.

(3) Touch the loop containing the bacteria to the drop of hydrogen peroxide and observe for *immediate bubbling.*

(4) Similarly test a known *Staphylococcus* sp to verify hydrogen peroxide activity.

d. **Interpretation:**

(1) Immediate bubbling in the drop of hydrogen peroxide signifies a positive test.

(2) Staphylococci are catalase-positive.

Absence of immediate bubbling signifies a negative test. Streptococci and pneumococci are catalse-negative.

(3) If the loop digs into blood agar medium, blood cells from blood agar may produce a false positive reaction.

(4) Platinum wire may produce a false positive reaction. Nichrome wire can be used satisfactorily.

14–10. Citrate Test:

a. **Principle.** This test is based on the ability of certain bacteria to utilize sodium citrate as the sole available source of carbon in a chemically defined medium. Alternately, the test can be performed in Koser citrate broth, which is essentially similar to Simmons except that it contains neither a dye indicator nor agar. Growth, evidenced by clouding of the broth, indicates a positive test in Koser broth. Some authorities feel that using a broth is the correct way to perform the test and that adding agar and indicator increases the unknowns in the test procedure. Christensen's citrate agar is a special preparation of limited application and is primarily used to differentiate *Shigella sonnei* and from anaerogenic *Escherichia* coli.

b. **Medium.** Simmon's citrate agar.

c. **Procedure and Interpretation.** Inoculate the slant of the medium with a straight wire or needle. The butt may also be stabbed if desired. Incubate at 35 °C for up to 4 days. Growth plus a green to blue color change in the medium indicates a positive test. Growth with no change in the medium indicates a positive test. No growth accompanied by no change in color indicates a negative test.

(1) *Koser Citrate Broth.* Inoculate with a needle or loop and incubate at 35 C for up to 4 days. Turbidity resulting from growth indicates a positive test. Medium remaining clear indicates a negative test.

(3) *Christensen Citrate Agar.* Inoculate slant with a needle or straight wire. Incubate at 35 C for 5 to 7 days. A positive citrate test is indicated by the appearance of a deep rose color. Blackening of the medium indicates H2S production. *Do not use as a routine citrate medium.* All organisms positive on Simmons or Koser are not positive on Christensen. However, all bacteria positive on Christensen are positive on the other media. Christensen citrate agar is used in the limited application of differentiating *Shigella sonnei* from the culturally similar, anaerogenic strains of enterobacteria called anaerogenic *Escherichia* coli.

d. **Discussion:**

(1) Tubes must be thoroughly cleaned and rinsed to remove traces of alkali and acid. The test is pH-dependent, and small amounts of either substance will alter the pH of the medium.

(2) If test results are equivocal, continue incubation for up to 7 days.

(3) Ideally inoculation should be made from saline suspensions; however, colonies can be taken directly from an agar plate if care is taken not to transfer any of the medium. Use a light inoculum if colonies are transferred directly.

(4) *Escherichia coli* is citrate-negative; *Klebsiella-Enterobacter* are positive.

14–11. Coagulase Test:

a. **Principle.** Coagulase activity is essentially confined to staphylococci and is related to the pathogenic species of staphylococci. Coagulase activity is demonstrated when a species of staphylococci capable of producing the enzyme coagulase is added to human or rabbit plasma and a thrombus or clot is formed. This procedure can be performed on a glass slide or in a test tube.

b. **Reagents:**

(1) Plasma, human or rabbit, fresh or rehydrated.

(2) Physiological saline.

c. **Technique:**

(1) *Slide Method:*

(a) Place a drop of physiological saline on a clean glass slide and prepare a rather heavy, even suspension of staphylococci in the drop of saline.

(b) Place a loopful of fresh or recently reconstituted dehydrated plasma in the suspension of staphylococci. Mix and then withdraw the loop.

(c) Immediately observe for the formation of a clot. This usually occurs within a few seconds with coagulase-positive species.

(2) *Tube Method:*

(a) Transfer 0.5 ml of a 24-hour broth culture of staphylococci or transfer a large loopful of growth from an agar plate of staphylococci to 0.5 ml of human or rabbit plasma in a glass tube.

(b) Incubate the tube at 37°C, preferably in a water bath for 3 hours.* Observe approximately every 30 minutes for clotting.

d. **Interpretation:**

(1) Pathogenic species of staphylococci

*Since a few strains do not clot within this period, the reading should be checked after 24 hours incubation.

usually give a positive reaction which is evidenced when the plasma is coagulated and produces a visible clot.

(2) In negative reactions the suspension remains homogeneous, and coagulation does not take place.

(3) A positive reaction, whether on a glass slide or in a tube, is decisive. A negative slide test must be confirmed by a tube test.

14–12. Cytochrome Oxidase Test for Enterobacteriaceae (Ewing and Johnson):

a. **Principle.** This test differentiates Gram-negative organisms (*Pseudomonas, Vibrio, Aeromonas*), which produce indophenol oxidase, from Enterobacteriaceae, which do not.

b. **Reagent. Prepare as follows:**

(1) *Solution A:*

Alpha-naphthol	1 g
95 percent Ethyl alcohol	100 ml

(2) *Solution B:*

Dimethyl-para-phenylenediamine dihydrochloride	1 g
Distilled or dionized water	100 ml

Store the reagents in the refrigerator in brown bottles. Discard solution B when it turns a dark color.

c. **Procedure and Interpretation:**

(1) Culture organisms on nutrient agar slants for 18 to 24 hours.

(2) Immediately prior to use, mix equal volumes of reagents A and B; for example, 1 ml A to 1 ml B.

(3) Add 2 to 3 drops of mixed reagents to the slant. Positive cultures develop a pink, a maroon and finally a blue color within 30 seconds to 1 minute.

(4) All Enterobacteriaceae are negative while *Pseudomonas, Vibrio, Aeromonas,* and *Alcaligenes* are positive.

14–13. Decarboxylase Tests—Arginine, Lysine, Ornithine:

a. **Principle:**

(1) Decarboxylase tests differentiate organisms on the basis of their ability to produce decarboxylases, which are proteolytic enzymes that degrade amino acids.

(2) In a decarboxylation, carboxyl groups within the amino acid molecule are removed and destroyed, producing in the process CO_2 and putrefactive amines (cadaverine, putrescine, etc).

(3) Organisms produce decarboxylases only at low (acid) pH and in the presence of peptone. These conditions are provided for in the test by culturing the organisms in a medium composed of peptone, dextrose, indicator, and amino acid compound. As growth occurs, organisms rapidly ferment the dextrose, producing the low pH necessary for formation of the enzyme, which in turn attacks the amino acid. As the amino acid is decarboxylated, the alkaline amine byproducts raise the pH of the medium, and the indicator is converted to an alkaline reaction color, which indicates a positive test.

b. **Procedure and Interpretation:**

(1) From an 18 to 24 hour slant culture, inoculate a control tube and a tube of each amino acid with a needle or wire through the mineral oil layer.

(2) Incubate at 37° C and examine daily for up to 4 days.

(3) In positive tests the medium first turns yellow due to acid production, then violet as decarboxylation occurs. A violet color is a positive reaction.

(4) The control should turn yellow and remain yellow.

c. **Discussion:**

(1) The use of a mineral oil seal is mandatory because necessary acidic conditions in the test are realized through fermentative (anaerobic) utilization of the glucose in the culture medium.

(2) Ornithine tests may show a small amount of floccular precipitation. This does not interfere with, or invalidate, the test.

(3) The arginine decarboxylase test can also be used to detect organisms producing ammonia through arginine dehydrolase conversion systems. To do this, 0.25 ml of Nessler's reagent is added to positive arginine tests on urease-negative organisms. The appearance of a brown color indicates the NH_3 in the medium resulted from an arginine dehydrolase conversion system.

(4) The Falkow decarboxylase method is not recommended for testing Family *Enterobacteriaceae* because of unsatisfactory results with *Klebsiella* and *Enterobacter*.

14–14. Ferric Chloride Test for Differentiation of Enterobacteriaceae:

a. **Principle.** Some members of the *Enterobacteriaceae* contain an enzyme which catalyzes the oxidative deamination of most of the L-amino acids to their corresponding alpha-ketoacids. When cell suspensions of organisms containing this enzyme are incubated with L-tryptophan (an L-amino acid), added ferric ions will react with the formed Indol-3-pyruvic acid (the corresponding alpha-keto acid) to

produce a very intense and stable cherry-red color.

b. **Reagents:**

(1) L-tryptophan, 0.2 percent.

(2) Ferric chloride, 10 percent.

(3) Hydrochloric acid, 50 percent.

c. **Technique:**

(1) To a Kahn tube containing 0.5 ml of the tryptophan solution, add approximately half the growth from a 24-hour TSI slant of the suspected organism.

(2) Shake this suspension for 1 hour on a Kahn shaking machine.

(3) Add one drop of 50 percent hydrochloric acid and one drop of 10 percent ferric chloride.

d. **Interpretation:**

(1) A very intense cherry-red color develops instantly with strains of the Proteus-Providence groups.

(2) All other *Enterobacteriaceae* develop a yellow color distinguishable from that of ferric chloride alone.

14-15. Ferric Chloride Test for Detecting the Hydrolysis of Sodium Hippurate:

a. **Principle.** Sodium hippurate is hydrolyzed to benzoic acid in the presence of Group B, beta hemolytic streptococci. The benzoic acid combines with the reagent to form an observable, permanent precipitate.

b. **Reagent and Medium:**

(1) Ferric chloride, 12 percent.

(2) Sodium hippurate broth, 1 percent.

c. **Technique:**

(1) Add 0.2 ml of the reagent to 0.8 ml of a 24-hour culture prepared in sodium hippurate broth.

(2) Mix immediately and observe after 10 to 15 minutes.

d. **Interpretation:**

(1) The formation of a permanent precipitate after approximately 10 to 15 minutes indicates a positive reaction.

(2) It is important to measure accurately the amounts of reagent and medium used in the test to prohibit the redissolving of benzoic acid in an excess of the reagent.

(3) If evaporation of the broth medium occurs during incubation, replace the evaporated volume with distilled water to restore the original concentration of sodium hippurate.

14-16. Gelatin Liquefaction Test:

a. **Principle.** Some bacteria possess the ability to produce proteolytic enzymes which can liquefy gelatin. Gelatin is a protein substance lacking essential amino acids and it is normally a liquid at 25°C or above.

b. **Medium.** Ordinary extract or infusion broth containing 10 to 15 percent gelatin.

c. **Technique:**

(1) Inoculate a tube of solidified (refrigerated) nutrient gelatin by stab technique.

(2) Incubate the inoculated tube and a control gelatin tube at 37°C, at which temperature the gelatin will liquefy.

(3) At the end of *each* 24-hour incubation period, place the inoculated tube and the control tube of liquefied gelatin in a refrigerator for a sufficient length of time to determine whether digestion of gelatin has occurred. Check the tubes for liquefaction in this manner for as long as 2 weeks unless digestion occurs sooner.

d. **Interpretation:**

(1) If a portion of gelatin liquefies, this indicates a positive test.

(2) If the gelatin remains solid, this indicates a negative test.

(3) The test is frequently used to distinguish the genus *Aerobacter* from other members of the *Enterobacteriaceae*, primarily the genus *Klebsiella*.

(4) Coagulase-positive staphylococci usually liquefy gelatin.

(5) An alternate procedure less frequently used is to proceed from step 1 to an incubation at 20°C, at which temperature the gelatin will remain solid. In this method, the shape of the portion of gelatin being liquefied can be observed. Incubation at 37°C appears to be more popular because the shape of the liquefied portion is less important than the time lost during the slower incubation at 20°C.

14-17. Gluconate (2-Ketogluconate) Oxidation Test:

a. **Principle.** The test differentiates on the basis of the ability of an organism to oxidize gluconate to 2-ketogluconate.

b. **Reagents:**

(1) **Gluconate Broth (Shaw and Clark).**

(2) Gluconate Substrate Tablets.

c. **Procedure and Interpretation:**

(1) Inoculate a tube of the medium with a loopful of an overnight broth culture and incubate at 37°C for 18 to 24 hours.

(2) Add a Clinitest urine sugar tablet to the tube and observe.

(3) The formation of a brown, tan, or orange precipitate indicates a positive test.

d. **Discussion:**

(1) If desired, Benedict's qualitative rea-

gent can be used instead of Clinitest. Use it by adding 1 ml to a culture and placing it in a boiling waterbath for 10 minutes. The test is positive when the precipitate is formed, as explained above.

(2) *Enterobacter aerogenes* and *Pseudomonas aeruginosa* give a positive test. *Escherichia coli* is negative.

14–18. Hydrogen Sulfide Test:

a. **Principle.** The production of hydrogen sulfide can be detected in bacterial cultures by observing blackening of the medium which is produced when hydrogen sulfide comes into contact with certain metals (for example, lead, iron, and bismuth) and produces the sulfides of these metals. In triple sugar iron (TSI) medium, ferrous sulfate is incorporated as an indicator for hydrogen sulfide production. An alternative procedure consists of testing a growing culture for hydrogen sulfide by using lead acetate paper. The latter technique is described below.

b. **Reagent.** Lead acetate, 10 percent.

c. **Technique:**

(1) Cut a sheet of filter paper into strips approximately 6 × 65 mm in size.

(2) Soak the strips of filter paper in 10 percent lead acetate solution and allow them to air dry.

(3) Autoclave the lead acetate paper strips at 15 lbs pressure for 15 minutes in petri dishes.

(4) Place one of the strips in the mouth of each culture tube so that a portion of the strip projects well below the cotton plug.

(5) Incubate the tubes at 37°C and examine daily to determine if blackening of the strip has occurred.

d. **Interpretation:**

(1) A positive test is indicated by blackening of the strip.

(2) No evidence of blackening of the strip indicates that the test reaction is negative.

(3) The filter paper technique is especially useful for detection of *Brucella* spp and for distinguishing among the species of *Brucella*. When an attempt is made to distinguish among the *Brucella* spp, the filter paper strips must be changed daily after the first appearance of blackening. The number of days a certain species of *Brucella* continues to produce hydrogen sulfide is quite significant for the identification of the species (see *Brucella*).

14–19. Indole Test:

a. **Principle.** This test is based on the ability of certain bacteria to split tryptophan to alanine and indole. The liberated indole will combine with para-dimethyl-amino benzaldehyde in Kovac's reagent to give a deep red color.

b. **Reagents and Media:**

(1) Kovac's reagent.

(2) Tryptophan broth.

(3) Trypticase nitrate broth.

c. **Technique:**

(1) Add 0.5 ml of Kovac's reagent to 3 to 5 ml of a 24 to 48 hour broth culture of the organism.

(2) Observe for the appearance of a red color at the interphase between the reagent and the broth culture.

d. **Interpretation:**

(1) The presence of the red color at the interphase between the reagent and the broth culture signifies that indole has been liberated.

(2) The absence of the red color signifies a negative test.

14–20. Kligler Iron Agar:

a. **Principle:**

(1) KIA is a primary differential medium and is used to presumptively differentiate Gram-negative bacteria. The most important use of the medium is to differentiate lactose-fermenting coliform bacteria (*Escherichia*, *Enterobacter*, etc) from the non-lactose-fermenting enteric pathogens (*Salmonella* and *Shigella*).

(2) KIA medium is basically a nutrient medium containing glucose, lactose, phenol red indicator, and iron salts. The medium differentiates on the basis of utilization of glucose and lactose with production of acid and sometimes gas. The iron salts provide a means of detecting H_2S.

(3) Glucose utilization (or fermentation) is a characteristic common to all enterobacteria; and most of the nonpathogenic enterics utilize lactose. The pathogenic enterobacteria, *Salmonella* and *Shigella* species, generally do not utilize lactose. Fermentation of glucose occurs anaerobically in the butt or depth of the tubed medium, and as acid is produced, the phenol red indicator changes to a yellow color. Fermentation of lactose occurs aerobically on the exposed slant area, and resulting acid production causes the slanted area to turn yellow. Most often, the slant area of the medium becomes red when lactose is not utilized. The appearance of the red color is due to alkaline reversion. Nonutilization of either carbohydrate can be indicated by appearance of the red color, but sometimes the medium will re-

main a neutral deep amber in this condition.

(4) Gas (H_2) production causes splitting or bubbling in the medium while H_2S is usually seen as blackening in the upper portion of the medium. Some species do not produce much H_2S, and only a small amount of blackening is seen just at the base of the slant.

(5) KIA reactions, their indications, and examples of organisms giving these recations are shown in Difco Manual p. 161.

b. **Reagent.** Klinger Iron Agar.

c. **Procedure and Interpretation:**

(1) Inoculate a tube of medium from a single isolated colony by stabbing the butt and, as the needle or wire is withdrawn, streaking the slant in a single operation. Preferably the inoculation material should be collected from the center of a single colony.

(2) Incubate at 35°C for 18 to 24 hours and examine without delay. Interpret as indicated below:

(a) Glucose fermented: alkaline slant, acid butt (ALK/A).

(b) Glusose and lactose fermented: alkaslant, acid butt (A/A).

(c) No carbohydrate fermented: alkaline slant, alkaline butt (ALK/ALK), or no change (NC/NC).

(d) H_2S: blackening in butt.

(e) Gas: disruption and / or bubbles in medium.

d. **Discussion:**

(1) Many workers prefer to use triple sugar iron agar (TSI); this medium is similar to KIA except that it contains sucrose as well as glucose and lactose. Inclusion of sucrose in TSI allows rapid differentiation of H_2S-producing *Salmonella* species from *Proteus vulgaris*, and H_2S-producing coliform organisms. The main disadvantage is that in many screening procedures for enteric pathogens all A/A cultures are discarded; consequently, many of the so-called "paracolors" of questionable pathogenicity are not detected. In KIA many of the "paracolors" give the ALK/A reaction expected of pathogenic *Salmonella* and *Shigella* species and are, therefore, noticed.

(2) Tubes used to contan the medium should have loose-fitting caps or cotton plugs so that there is a free exchange of air in the tube. If screw-capped tubes are used, the caps must be loosened prior to incubating the culture.

(3) Gas production is sometimes difficult to detect. When in doubt, use a carbohydrate broth containing a Durham insert for gas detection.

14–21. Lancefield Precipitin Test for Grouping and Typing of Beta Streptococci:

a. **Principle.** The group or Type of a beta streptococcus can be determined by combining prepared antigen with various prepared antisera until precipitation is observed in the solution of prepared antigen and the specific antiserum.

b. **Reagents and Media:**

(1) Hydrochloric acid, N/5.

(2) Sodium hydroxide, N/5, buffered.

(3) Phenol red indicator, 0.01 percent.

(4) Thymol blue indicator, 0.01 percent.

(5) Beta streptococcus grouping and typing serum.

(6) Tryptose phosphate broth.

(7) Todd-Hewitt broth.

c. **Technique for Preparation of Antigen by the Hot Hydrochloric Acid Method:**

(1) Inoculate the organisms to tryptose phosphate broth or Todd-Hewitt broth and incubate for 18 to 24 hours at 37°C.

(2) Centrifuge the broth culture and discard the supernate.

(3) Resuspend the remaining sediment in 0.4 ml of N/5 hydrochloric acid. Mix thoroughly.

(4) Add one drop of thymol blue indicator. An orange-red or peach color should result.

(5) Heat the suspension in a boiling water bath for 10 minutes.

(6) Cool the suspension in a cold water bath or refrigerator for 10 minutes.

(7) Centrifuge and decant the supernate to a clean tube.

(8) Add one drop of phenol red indicator. A distinct yellow color should result.

(9) Add buffered sodium hydroxide dropwise until a pale pink color develops.

(10) Centrifuge and carefully remove the supernate which, if clear, is ready for the precipitin test. Turbid supernates interfere with the reading of the test and are unsuitable for use.

d. **Technique for Preparation of Antigen by the Autoclave Method:**

(1) Inoculate the organisms to tryptose phosphate broth and incubate for 18 to 24 0.5 ml of physiological saline. Mix thoroughly.

(2) Centrifuge the broth culture and discard the supernate.

(3) Resuspend the remaining sediment in hours at 37°C.

(4) Autoclave the tube for 15 minutes at 15 pounds pressure.

(5) Centrifuge and carefully remove the supernate which, if clear, is ready for use in

the precipitin test. Turbid supernates interfere with the reading of the test and are unsuitable for use.

e. **Technique for Group Precipitin Test Using Capillary Tube Method:**

(1) Dip a clean capillary tube about 2 inches in length and 1.0 to 1.5 mm in diameter into group A streptococcal antiserum and draw up the antiserum to approximately one-third the length of the tube.

(2) Wipe the excess antiserum from the antigen and draw up an equal amount of the outside of the capillary tube.

(3) Dip the capillary tube into prepared antigen in such a manner that *no* air space separates the two solutions.

(4) Wipe the excess antigen from the outside of the capillary tube.

(5) Allow the column of liquid to flow to the central portion of the tube, leaving an air space at each end of the column of liquid.

(6) Insert the capillary tube into a plasticine block and observe for the formation of a precipitate after 15 to 30 minutes.

(7) In succession, repeat the above procedure using beta streptococcal grouping antisera (groups B, C, D, E, F, and G) until precipitation occurs. A normal serum control should be included.

f. **Technique for Group Precipitin Test by the Small Tube Method:**

(1) Place 0.5 ml of the desired streptococcal antiserum into a small tube.

(2) Add an equal amount of the prepared antigen.

(3) Prepare a normal serum control.

(4) Observe after 15 to 30 minutes for a precipitate.

g. **Technique for the Type Precipitin Test:**

(1) Use the same technique as described for the group precipitin test substituting beta streptococcal *typing* serum in place of the beta streptococcal *grouping* serum.

(2) Incubate the tubes for 2 hours and observe for precipitation.

(3) If no precipitation is observed after the incubation period of 2 hours, refrigerate the tubes overnight and then observe for precipitation.

h. **Interpretation:**

(1) The antigen prepared by the hot hydrochloric acid method or by the autoclave method will produce a precipitate in 15 to 30 minutes with the group-specific antiserum or in 2 to 24 hours with the type-specific antiserum.

(2) Since the type-specific M substance is lost after cultivation on artificial media, typing should be performed as soon as possible.

14–22. Lysine Decarboxylase Differential Test:

a. **Principle.** Certain members of the *Enterobacteriaceae* can be differentiated on the basis of their ability to decarboxylate some amino acids. Lysine decarboxylase broth (Falkow modification) is a differential medium recommended for distinguishing *Salmonella* and *Arizona* from the *Citrobacter* group, and paracolons resembling shigellae from the pathogenic *Shigella*.

b. **Medium.** Lysine decarboxylase broth.

c. **Technique:**

(1) Culture the test organisms in lysine decarboxylase broth for 18 to 24 hours at 37° C. Record results.

(2) Inoculate a control tube (decarboxylase basal broth) for each culture being investigated.

(3) A positive test is indicated by a purple color. The control tube should remain yellow.

(4) If test organisms are negative (yellow), prolong the incubation and examine daily for 4 days before discarding as negative.

d. **Interpretation:**

(1) The sterile broth, pH 6.5, is purple.

(2) The medium first becomes yellow because of acid production from glucose. If decarboxylation occurs later, the medium becomes purple again, or more alkaline. Some positive cultures give latent reactions, for example, within the 4-day period. The basal decarboxylase medium can be obtained for addition of other amino acids.

14–23. Malonate Utilization Test:

a. **Principle.** The malonate test differentiates on the basis of an organism's ability to utilize sodium malonate as a sole source of carbon.

b. **Reagents.** Malonate Phenylalanine Medium

c. **Procedure and Interpretation:**

(1) Inoculate the medium and incubate at 37° C for 24 hours.

(2) A positive malonate test is indicated when the culture turns a deep blue color.

(3) A negative test is indicated by an unchanged, greenish-colored culture.

(4) The phenylalanine deaminase test is performed by acidifying the broth with 0.1 to 0.2 ml of 0.1N HCl and adding 0.1 ml of 10 percent aqueous ferric chloride. Mix well and observe immediately for formation of a green color which fades rapidly.

d. **Discussion.** The malonate test is useful

in differentiating *Arizona* and *Salmonella*. Most Arizona strains, *Enterobacter* and *Klebsiella* give a positive malonate test. *Escherichia* and *Salmonella* are negative.

14-24. Methylene Blue Reduction Test for Enterococci:

a. **Principle.** Some bacteria have the ability to decrease the oxidation-reduction potential of a medium as they multiply, consuming oxygen dissolved in the medium. Methylene blue, an oxidation-reduction indicator, is added to the medium to indicate a change in the oxidation-reduction potential.

b. **Medium.** Methylene blue milk medium, 0.02 percent methylene blue.

c. **Procedure:**

(1) Inoculate a tube containing approximately 10 ml of methylene blue milk medium with the streptococcal organisms to be tested.

(2) Incubate the medium at 37°C and observe for a color change which may take place within a few hours.

d. **Interpretation:**

(1) Streptococci of enterococcal origin will reduce the methylene blue medium to its colorless state within 24-hours incubation.

(2) Streptococci of other than enterococcal origin will not decolorize the original blue color of the methylene blue solution.

(3) The enterococcal group may be differentiated from other streptococci on the basis of the ability of the enterococci to reduce the medium, changing the methylene blue from its blue color (oxidized form) to its colorless state (reduced form).

14-25. Methyl Red Test:

a. **Principle.** Some organisms ferment glucose-producing acids which are then converted to neutral end products. This test is designed to differentiate those organisms which, in contrast, produce stable acid end-products.

b. **Reagent and Medium:**

(1) Methyl red reagent.

(2) Clark and Lub's broth (MR-VP medium).

c. **Technique.** Add about 5 drops of methyl red indicator to a 5-ml portion of a 2- to 7-day old culture, prepared in Clark and Lub's broth.

d. **Interpretation:**

(1) A positive reaction occurs when the culture is sufficiently acid to turn the methyl red reagent a distinct red color.

(2) A yellow color is regarded as a negative test.

14-26. Motility Test:

a. **Principle:**

(1) The purpose of the test is to determine whether organisms are motile or nonmotile.

(2) The procedure described below is that which is commonly used to examine enterobacteria. Fastidious organisms may require enriched media, and they may have special temperature requirements.

b. **Reagent.** Motility Test Medium.

c. **Procedure and Interpretation.** Inoculate the medium by stabbing with a straight wire or needle to a depth of one-half the length of the butt. Incubate at 35°C for 18 to 24 hours. Motile organisms spread out and away from the line of inoculation and show cloudy diffuse growth. Nonmotile organisms remain along the line of inoculation in compact nonspreading growth.

d. **Discussion:**

(1) The flagellar activity of certain organisms is adversely affected by incubation at 35°C; therefore, motility test medium should be incubated at temperatures near 18°C to 20°C when negative results are obtained with organisms suspected or known to be motile.

(2) Motility can be observed microscopically using hanging drop and darkfield techniques. Look for directional movement.

(3) Young cultures, preferably in the logarithmic phase, should be used for motility testing. Broth cultures are recommended as an inoculum.

(4) Many motile organisms cease flagellar activity as the cultures become acid.

(5) Strict anaerobes lose motility on exposure to oxygen in the atmosphere.

(6) In wet mount preparations strict aerobes will migrate to the periphery of the specimen due to the availability of a high oxygen concentration.

14-27. Ninhydrin Test:

a. **Principle.** Some members of the *Enterobacteriaceae* have the ability to decarboxylate amino acids. This process is followed by changes in pH which can be demonstrated by use of an indicator. In this test the lysine in pancreatic digest of casein is decarboxylated to cadaverine, which, when extracted with chloroform, reacts with the ninhydrin indicator

b. **Reagents and Medium:**

(1) Sodium hydroxide, 4 N.

(2) Chloroform.

(3) Ninhydrin reagent.

(4) Carlquist ninhydrin base.

c. **Technique:**

(1) Inoculate the medium from broth or agar and incubate at 37°C for 18 to 24 hours.

(2) After incubation, add 1 ml of 4 N sodium hydroxide and mix well.

(3) Add 1 ml of chloroform, shake the tube vigorously, and centrifuge the tube for 10 minutes at 2,000 rpm.

(4) Transfer 0.5 ml of the chloroform extract to a test tube. Exercise care to secure only the chloroform extract below the layer of denatured protein.

(5) Add 0.5 ml of ninhydrin reagent and allow this mixture to stand at room temperature for 4 minutes.

d. **Interpretation:**

(1) A positive reaction is indicated by the development of a deep purple color.

(2) No color change indicates a negative reaction.

(3) This test is positive for *Salmonellae* (other than *S. paratyphi* A), *E. coli*, and *Klebsiella* and *Aerobacter* spp. A negative reaction occurs with Providencia and Bethesda-Ballerup groups. *Shingella* spp, and *S. paratyphi* A. *Proteus* spp are irregular in their reaction.

14–28. Nitrate Reduction Test:

a. **Principle.** Bacteria are grown in a broth culture containing potassium nitrate to determine the ability of the bacteria to reduce nitrate to nitrite.

b. **Reagents and Medium:**

(1) Alpha-naphthylamine.

(2) Sulfanilic acid.

(3) Peptone water containing 0.001 to 0.1 percent potassium nitrate (37).

c. **Procedure:**

(1) Add 1 ml of sulfanilic acid to approximately 5 ml of a 24-hour culture grown in peptone broth containing 0.02 percent potassium nitrate.

(2) Add, dropwise, 1 ml of alphanaphthylamine and observe for a light pink to deep red color.

d. **Interpretation:**

(1) The presence of a light pink to deep red color indicates that nitrate has been reduced to nitrite by the bacteria being tested.

(2) The absence of color indicates the absence of nitrite, which could be the result of nonreduction of nitrate or reduction of both nitrate and nitrite.

(3) To determine if both nitrate and nitrite have been reduced, add a small amount of zinc dust to the colorless mixture of culture and reagents.

(4) The presence of a red color indicates that zinc has reduced nitrate to nitrite, thereby indicating that nitrate was present. Nitrate would be present if it were not reduced by bacterial action.

(5) The absence of a red color indicates that the bacteria have reduced nitrate to nitrite and then further reduced the nitrite.

14–29. ONPG (β-*D*-Galactosidase) Test:

a. **Principle:**

(1) The test demonstrates that certain organisms can be induced to produce the enzyme β-galactosidase by culturing them on a medium containing a galactoside, for example, lactose.

(2) In the test, organisms cultured on a lactose medium (for example, KIA, or TSI) are incubated in a solution containing *o*-nitrophenyl-β-D-galactopyranoside (ONPG). If β-galactosidase has been induced and is present in the organisms, ONPG will be converted from colorless *o*-nitrophenyl compound to a yellow *o*-nitrophenol compound.

b. **Reagents.** *ONPG-Peptone Water (Lowe)*:

c. **Procedure and Interpretation:**

(1) Emulsify a loopful of growth from KIA or TSI medium in 0.25 ml of normal saline.

(2) Inoculate a loopful to a tube of ONPG-peptone medium or a reconstituted, commercially prepared ONPG tablet.

(3) Incubate ONPG-peptone medium weight overnight in an incubator. Incubate ONPG reconstituted tablet medium 6 hours at 37°C.

(4) Appearance of a yellow color (colorless → yellow) indicates *o*-nitrophenyl has been converted to *o*-nitrophenol, and the test is positive. Many organisms give positive tests in 3 to 6 hours if a heavy inoculum is used. Discard the test after 24 hours.

d. **Discussion:**

(1) A lactose-positive organism will also be ONPG-positive; however, an organism can be ONPG-positive and a nonlactose-fermenter or both ONPG- and lactose-negative.

(2) The test is useful in differentiating *Salmonella* (a nonlactose-fermenter) from *Citrobacter* and *Arizona* (late or slow lactose fermenters) and in studying several nonenteric organisms.

14–30. Oxidase Test:

a. **Principle.** This test is based on the production of an oxidase enzyme by the members of the genus *Neisseria*. Upon the addition of

oxidase reagent to growth of a *Neisseria* sp, the presence of oxidase enzyme is evidenced by a color change in the colonies.

b. **Reagents.** Dimethyl-p-phenylenediamine hydrochloride (para-aminodimethylaniline) or tetramethyl-p-phenylene diamine hydrochloride.

c. **Technique:**

(1) Add 2 to 3 drops of the oxidase reagents to surface colonies appearing to be *Neisseria* spp on an agar plate.

(2) Colonies of oxidase-positive organisms first become pink, then maroon, through purple, to black.

d. **Interpretation:**

(1) Colonies of *Neisseria* species should show the color change; discoloration of the surrounding medium is not significant. Within 1 to 2 minutes the oxidase-positive colonies become black if the dimethyl reagent is used, or purple if the tetramethyl reagent is used.

(2) Perform the Gram-stain technique on all oxidase-positive (black or purple) colonies, because some organisms other than *Neisseria* spp will give the positive oxidase reaction.

(3) Organisms most likely to cause confusion because of their oxidase-positive reactions are *Alcaligenes*, *Hemophilus*, and some of the Gram-negative coccoid organisms discussed in Chapter 6.

(4) False negative reactions may occur when *Pseudomonas* spp grow in close proximity to *Neisseria* colonies. *Pseudomonas* spp produce a substance which completely inhibits the production of oxidase by *Neisseria* spp.

14–31. Oxidation-Fermentation Test (Hugh and Leifson):

a. **Principle:**

(1) The O-F test determines whether organisms break down energy-yielding substances oxidatively (aerobically) or fermentatively (anaerobically).

(2) In the test, organisms are cultured simultaneously in two tubes of a sugar medium. One tube is overlaid with mineral oil or paraffin to provide an airtight seal, and the other is left open or exposed. An organism that utilizes the sugar oxidatively produces a color change or acid in the open tube only. Fermentative organisms produce a color change or acid in both tubes. Nonutilizers produce slight or no change. In some cases, nonutilizers produce an alkaline (blue) color change, which is due to degradation of the peptone in the medium.

(3) The O-F test is most often performed with glucose as the sugar supplement; however, other sugars are also used and are of value in separating closely related species.

b. **Reagent.** *O-F Medium (Hugh-Leifson):* Dissolve the solids in water with heat. Adjust the pH and add the indicator. Sterilize at 121 C for 15 minutes. Cool and add a sterile solution of the desired carbohydrate to give a final concentration of 1 percent, for example, if glucose is used, prepare a 10 percent solution, filter sterilize, and add 10 ml per 100 ml of basal medium. Mix the carbohydrate and basal medium and dispense 10 ml per 16 mm or less in a cotton plugged tube.

c. **Procedure and Interpretation:**

(1) Using a straight wire or needle, inoculate two tubes of medium with organisms from a young slant culture.

(2) Overlay one tube with about 1 cm of sterile mineral oil.

(3) Incubate both tubes at 37°C for 48 hours or longer. Make an initial reading after 48 hours. Discard after 14 days.

Report	*Open*	*Closed*
Oxidation	yellow	green
Fermentation	yellow	yellow
Nonutilization	blue or green	green

d. **Discussion:**

(1) If desired, the following organisms can be used as controls:

(a) Oxidative—*Acinetobacter anitratus.*

(b) Fermentative—*Escherichia coli.*

(c) Nonutilization—*Alcaligenes faecalis.*

(2) Absence of change in the medium may be due to the following:

(a) The medium may not support growth of the organism.

(b) The organism may not utilize that specific carbohydrate.

(c) The organism may not utilize any carbohydrate.

(3) Some workers classify and interpret results of O-F tests as follows:

(a) Group I: Nonoxidizers, nonfermenters—no change in medium.

(b) Group II: Oxidizers, nonfermenters—acid in open tube only.

(c) Group IIIa: Fermenters (anaerogenic)—acid in both tubes.

(d) Group IIIb: Fermenters (aerogenic)—acid and gas in both tubes.

(e) Group IIIc: Fermenters and oxidizers—acid and gas in both tubes of one sugar but only acid in the open tube of another.

14–32. Potassium Cyanide (KCN) Test:

a. **Principle:**

(1) The KCN test differentiates bacteria on the basis of their ability to grow in a me-

dium containing potassium cyanide, a highly toxic and poisonous substance.

(2) The test is useful in differentiating *Salmonella* and *Shigella* organisms, which do not grow in KCN medium, from other enterobacteria which do grow in the medium.

b. **Reagents:**

(1) 0.5 percent Potassium Cyanide Solution.

(2) KCN Broth Base.

c. **Procedure and Interpretation:**

(1) Inoculate the test and controls with one loopful of an overnight broth culture. Tightly insert the cork or alternatively, tighten the screwcap and incubate at 37°C for up to 48 hours.

(2) Examine daily for evidence of turbidity which indicates growth.

(3) Growth is considered a positive test. The procedure should be run with known controls. *Escherichia coli* is KCN negative; *Enterobacter aerogenes* is KCN positive.

d. **Discussion:**

(1) Among Family Enterobacteriaceae, *Shigella*, *Escherichia*, *Salmonella*, *Edwardsiella*, and *Arizona* are KCN negative.

(2) *Enterobacter*, *Citrobacter*, *Hafnia*, *Proteus*, *Providencia*, and *Serratia* are KCN positive.

(3) Do not inoculate too heavily or the test may be falsely interpreted as positive.

(4) Dispose of used cultures by adding a crystal of ferrous sulfate ($feSO_4$) and 0.1 ml 40 percent potassium hydroxide (KOH) to each tube before autoclaving.

14–33. Phenylalanine Deaminase Test:

a. **Principle:**

(1) Deaminase tests in general are based on the fact that some bacteria produce enzymes called deaminases, which are capable of converting large amino acid molecules to simpler, smaller-sized molecules. Deaminated amino acids yield amines and ammonia.

(2) For example, in the phenylalanine deaminase test organisms are grown in a medium containing DL-phenylalanine is converted as follows:

$$\text{Phenylalanine} + \tfrac{1}{2}\,O_2 \xrightarrow{\text{(deaminase)}} \text{phenylpyruvic acid} + NH_3 \uparrow$$

The presence of the deaminated byproduct is detected by treating the culture with a ferric chloride reagent, which produces a characteristic green color, as follows:

$$\text{Phenylpyruvic acid} + Fe^{+++} \xrightarrow{\text{(acid)}} \text{Green Color}$$

(3) The phenylalanine deaminase test is particularly useful in differentiating enteric bacteria. Two species, *Proteus* and *Providencia*, are positive while all other enterics are negative.

b. **Reagents:**

(1) *Phenylalanine Agar* (*Ewing, Davis, and Reavis*).

(2) 10 percent *Ferric Chloride Reagent*.

c. **Procedure and Interpretation:**

(1) Inoculate the surface of the slant with a moderately heavy inoculum from an agar slant culture.

(2) Incubate at 37°C for 18 to 24 hours.

(3) Rotate the culture to loosen the agar; then add 0.2 ml 10 percent ferric chloride.

(4) A positive test is indicated by appearance of a green color on the slant and in the liquid at the base of the slant.

d. **Discussion:**

(1) *Pseudomonas aeruginosa* growths may yield blue to bluish-green color before the addition of ferric chloride reagent. The color is due to pyocyanin and fluorescein pigments naturally produced by many *P. aeruginosa* strains.

(2) Several media are available for performing the test. Some workers use a sodium malonate-phenylalanine medium and perform both a malonate test and deaminase test on a single culture. A rapid micro method (Henriksen) can be performed by suspending the entire growth on an agar slant in 0.5 ml of saline and adding 0.2 ml of the suspension to an equal volume of 0.2 percent saline solution of DL-phenylalanine. The mixture is incubated at 37°C for 4 hours with the tube placed in an almost horizontal position. Then 1 to 2 drops of phenol red is added, and the mixture is acidified with 10 percent H_2SO_4 until yellow. Next add 4 to 5 drops of a half-saturated solution of ammonium ferrous sulfate [$FeSO_4 \cdot (NH_4)_2 SO_4 \cdot 6H_2O$]. Shake and observe closely for the appearance of a green color within 1 minute. The color fades rapidly.

14–34. Pyrogallol Anaerobic Techniques:

a. **Principle.** When pyrogallic acid and sodium carbonate are mixed in an airtight system, oxygen is absorbed by alkaline pyrogallol, and an anaerobic atmosphere prevails. This technique can be modified for any leakproof vessel which will accommodate cultures. Modifications for the Bray and Petri dishes are described below.

b. **Regents and Medium:**

(1) Pyrogallic acid, 40 percent solution or reagent crystals.

(2) Sodium carbonate, 20 percent solution or reagent crystals.

(3) Blood agar or suitable anaerobic culture medium.

c. **Technique for Bray Dish:**

(1) Charge one side of the divided Bray dish bottom with 4 ml of a 40 percent solution of pyrogallic acid.

(2) Add 10 ml of a 20 percent solution of sodium carbonate to the other side of the Bray dish bottom. Care should be taken to prevent mixing of the solutions.

(3) Streak a blood agar plate or another suitable medium with the specimen or cultural growth of the anaerobic organism. The plate should be either the standard-sized, thin-bottomed, glass variety, or a standard Petri dish.

(4) Invert the inoculated agar dish and place it over the wide mouth of the Bray dish.

(5) Carefully seal the dish bottom to the Bray dish with a strip of parafilm, or suitable sealing material.

(6) Gently rock the vessel, thoroughly mixing the pyrogallic acid and sodium carbonate solutions.

(7) Place the culture in the incubator.

(8) After incubation (usually for 48 hours), remove the culture from the Bray dish and place a sterile Petri dish top over the agar so aseptic examinations of growth may be performed.

d. **Technique for Petri Dish:**

(1) Place lid of Petri dish on bench with inside up and insert section of filter paper.

(2) Place a pinch (approximately 0.5 gm) of crystalline pyrogallic acid and a pinch of crystalline sodium carbonate, together, on the filter paper. Mix. Moisture for the reaction will be obtained from the agar.

(3) Invert the bottom of the agar dish, containing the inoculated agar, and place it within the lid.

(4) Carefully seal the dish bottom to the lid with suitable sealing material.

(5) Continue with Step 7 of the Technique for Bray Dish.

e. **Discussion.** Cultures must be incubated in the usual inverted position so that the reagents or filter paper will not contaminate the culture. When several tubes or plates are to be incubated anaerobically in a large, leakproof vessel, the usual proportions of reagents are 10 gm of pyrogallic acid and 100 ml of 20 percent sodium carbonate per liter of air space.

14–35. Sellers Differential Test:

a. **Principle.** Gram-negative enteric bacilli, or coccabacilli, which produce alkaline reactions on TSI (or KIA) are subcultured to Sellers differential agar, recommended for differentiating certain nonfermentative Gram-negative organisms.

b. **Reagents and Medium:**

(1) Glucose solution, 50 percent, sterile.

(2) Sellers differential agar.

c. **Technique:**

(1) Prepare Sellers agar in large tubes (15 × 100 mm), slanted to give approximately 1.5-inch butts and 3-inch slants.

(2) Immediately before inoculation add two large drops of a sterile 50 percent glucose solution to each tube. Let these drops run down the side of the tube opposite the slant.

(3) Using a wire bacteriology needle, pick an isolated colony, or portion of growth on slant.

(4) Inoculate Sellers agar by stabbing the butt (deep) and streaking the slant as the needle is withdrawn.

(5) Incubate the agar for 18 to 24 hours at 37°C.

d. **Interpretation.** After an 18 to 24 hour incubation period, examine Sellers differential agar for the reactions in the slant (Fluorescence or Blue-color); the band area (oxidation of glucose) and the butt (alkalinization).

14–36. Streptococcus (Beta) Isolation and Identification:

a. **Principle.** The blood agar streak plate procedure is an acceptable and efficient procedure for the isolation of beta hemolytic streptococci. Techniques are included in this section to reduce the amount of variability inherent in the streak plate procedure.

b. **Reagents and Media:**

(1) Filter paper strips, sterile.

(2) Bacitracin discs, 1 or 2 units.

(3) Sodium chloride, 6.5 percent.

(4) Penicillin.

(5) Penicillinase.

(6) Blood agar (sheep cells preferred).

(7) Brain heart infusion broth.

c. **Technique for Filter Paper Transport:**

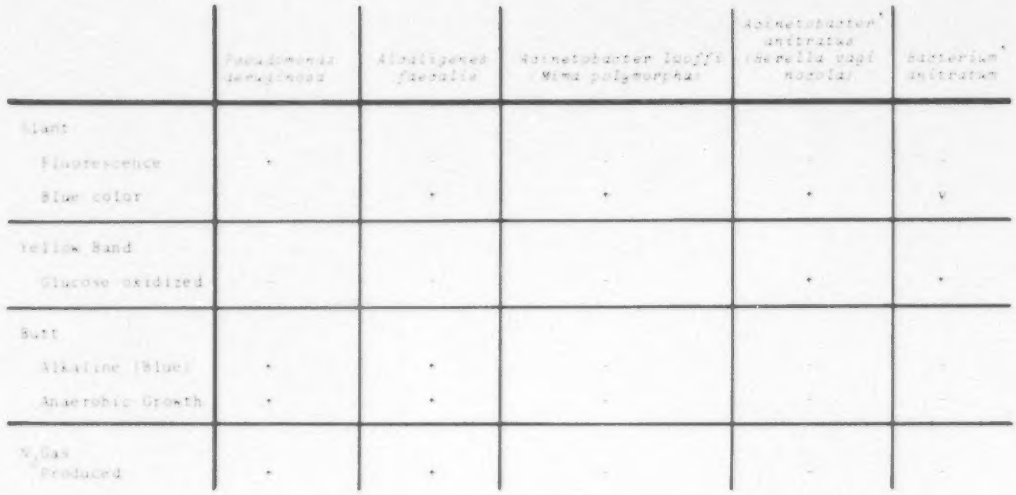
(1) Swab throat or nasopharynx with cotton swab.

(2) Rub swab over a sterile strip of filter paper (available commercially packaged).

(3) Dry the inoculated filter paper for 1 to 2 minutes and place in a sterile container.

(4) The specimen may be mailed or carried to the laboratory in the sterile container.

(5) At the laboratory, the strip is asep-



* Note: A. antitratus produces acid in dulcitol; B. antitratum does not.

Figure 14–1. Differentiation on Seller's Agar.

tically removed (sterile forceps) from the container and placed inoculum-side down (face down) on the surface of a blood agar plate.

(6) The plate and adhering filter paper are incubated for at least 4 hours, or overnight, at 37°C, CO_2.

(7) Following this initial incubation, aseptically remove the filter paper and streak the plate for isolation, using a wire loop.

(8) Incubate the plate for an additional 18 to 24 hours at 37°C, CO_2. Record results.

d. **Discussion.** When streaking the surface of a blood agar plate, either break the surface with a stab of inoculum or cover the inoculated areas with a sterile cover slip (pressed firmly) in order to demonstrate hemolysis resulting from oxygen-labile streptolysin "O".

e. **Technique for Bacitracin Screen Test:**

(1) Streak a blood agar plate with a pure culture of the beta hemolytic streptococci. Inoculation must be dense as for sensitivity studies.

(2) Place a .02 to .04 unit disc of bacitracin on the freshly streaked area of the plate.

(3) Incubate the plate for 18 to 24 hours at 37°C, CO_2.

f. **Discussion:**

(1) Of those beta hemolytic streptococci showing *sensitivity* to a .02 to .04 unit disc of bacitracin, approximately 85 percent will be Lancefield Group A streptococci.

(2) Of those beta hemolytic streptococci showing *resistance* to a .02 to .04 unit disc of bacitracin, approximately 100 percent will not be Lancefield Group A streptococci.

(3) This is a screening test not intended to provide final confirmation of Group A streptococci.

g. **Technique for Negative Enrichment:**

(1) Swab throat or nasopharynx with cotton swab.

(2) Immediately place this swab in 5 ml of brain heart infusion broth which contains 6.5 percent sodium chloride and 20 units/ml of penicillin (available commercially combined).

(3) Agitate the swab-broth tube gently and incubate for 2 hours at 37°C.

(4) Following incubation, reagitate this swab-broth tube.

(5) Transfer 0.1 ml of the incubated suspension to the surface of a blood agar plate, which has been *previously* overlaid with 0.2 ml of penicillinase.

(6) Spread the penicillinase-inoculum mixture evenly over the surface of the blood agar plate with a sterile glass rod and incubate for 18 to 24 hours at 37°C.

h. **Discussion.** Nonstreptococcal organisms multiplying in the salt concentration are eliminated by the penicillin, while beta hemolytic streptococci are inhibited and remain dormant. On the blood agar plate containing penicillinase, beta hemolytic streptococci resume growth without competition.

14–37. Triple Sugar Iron Test:

a. **Principle.** Following inoculation and incubation of the organism on TSI medium, sugar fermentation is evidenced by conversion of all

or portions of the medium from orange-red to yellow. The acid-end products of such fermentations convert phenol red to its yellow acid state. Those organisms failing to ferment a particular sugar(s) will either produce no change in the medium or convert it to a deeper red color. The latter reaction is a result of the action of alkaline metabolic end-products upon phenol red. TSI agar also contains sodium thiosulfate and ferrous ammonium sulfate to serve as indicators of hydrogen sulfide production. Organisms capable of producing hydrogen sulfide will do so through the utilization of sodium thiosulfate in an acid environment. Hydrogen sulfide, formed in this manner, will react with ferrous ammonium sulfate to yield ferrous sulfide. The latter substance accumulates as a black precipitate either immediately under the base of the slant or deeper in the butt of the TSI agar.

b. **Medium.** Triple sugar iron agar.

c. **Technique:**

(1) Using a wire bacteriology needle, pick an isolated colony for inoculation.

(2) Inoculate the TSI medium by stabbing the butt and streaking the slant as the needle is withdrawn.

(3) Incubate the agar for 18 to 24 hours at 37° C.

d. **Interpretation:**

(1) After an 18 to 24 hour incubation period, examine the TSI medium for the following reactions:

(a) Lactose fermentation—acid (yellow) reaction throughout the medium.

(b) Sucrose fermentation—acid (yellow) reaction throughout the medium.

(c) Glucose fermentation—acid (yellow) in butt of agar medium only, with an alkaline (dark red) slant. NOTE: In short incubation periods, glucose-fermenting organisms may produce an initial acid slant until the low concentration of glucose is consumed. Then under aerobic conditions the reaction reverts and becomes alkaline; alkaline end-products from the metabolism of proteins turn the indicator red in the aerobic environment (slant). No color change occurs in the anaerobic environment (butt).

(d) No sugars fermented—alkaline (dark red) slant with orange-red butt.

(e) Gas production from lactose, sucrose, or glucose fermentation—bubble formation or splitting of the medium in the butt of the tube.

(f) Hydrogen sulfide production—blackening in the butt or the area of the medium just under the base of the slant. Hydrogen sulfide will be produced only in an acid environment created by fermentation of at least one of the three sugars.

(2) In *routine examination* of fecal materials, all TSI slants with acid butts and alkaline slants should be retained for examination. Those which are acid throughout (possible *E. coli* or other coliform bacilli) may be discarded as nonpathogens. The TSI slants with alkaline slants and neutral butts may also be discarded when dealing with routine fecal specimens. The latter recations are indicative of *Pseudomonas* spp and *Alcaligenes* spp which are usually nonpathogens when present in the intestinal tract.

14–38. Urease Test:

a. **Principle.** The splitting of urea to ammonia and carbon dioxide by the enzyme urease may be employed as a reliable test to differentiate *Proteus* spp from other members of the enteric group. *Proteus* spp hydrolyze urea rapidly and release ammonia, which is indicated by a reddening of the indicator phenol red.

b. **Medium:**

(1) Christensen's urea agar.

(2) Urea broth (Rustigian and Stuart).

c. **Technique:**

(1) Transfer a heavy inoculum to the urea medium.

(2) Incubate urea agar for 2 to 5 hours; urea broth for 18 to 24 hours.

d. **Interpretation:**

(1) Urease production by the organism under study is evidenced by the medium's being converted from light amber to pink.

(2) In a negative reaction, no color change takes place.

(3) Many organisms cause Christensen's urea agar to turn soon after the medium is inoculated. In order for the test results to be valid for *Proteus* spp, the medium *must* become alkaline (pink color) within the first 2 to 4 hour interval of incubation.

(4) *Escherichia freundii* as well as certain members of the so-called Bethesda-Ballerup group of paracolons may convert Christensen's urea agar within 24 to 48 hours. *Klebsiella pneumoniae* also may produce a definite pink color in the medium after 18 to 24 hours incubation.

(5) Urea broth (Rustigian and Stuart) will be converted to the alkaline (pink) color by *Proteus* spp within 18 to 24 hours. This

medium will not detect smaller amounts of urease produced by certain species of coliform and paracolon bacilli.

14–39. Voges-Proskauer Test:

a. **Principle.** This test is based on the ability of some organisms to produce a neutral end-product, acetyl-methyl-carbinol, from dextrose.

b. **Reagents and Medium:**

(1) Alpha naphthol, 5 percent.

(2) Sodium or potassium hydroxide, 40 percent.

(3) Clark and Lub's broth (MR-VP medium).

c. **Technique:**

(1) Add 0.6 ml of 5 percent alpha naphthol in absolute ethyl alcohol to 1 ml of a 24- to 48-hour broth culture prepared in Clark and Lub's broth.

(2) To the same culture, add 0.2 ml of 40 percent sodium or potassium hydroxide.

(3) Shake the tube and allow it to stand for 5 to 10 minutes before interpreting results.

d. **Interpretation:**

(1) A positive test is indicated by the development of a pink or red color.

(2) A yellow color is regarded as a negative test.

(3) Occasionally, some difficulty is experienced in obtaining a positive V-P result with organisms which should produce acetyl-methyl-carbinol. This may be overcome by gently heating the culture after adding the test reagents. Strains which do not produce acetyl-methyl-carbinol (VP-negative) will still yield a negative result despite the application of gentle heat. Inoculate surface of the agar with a portion on a colony to be tested. Incubate for 3 days at 37°C. After incubation, overlay the culture with 0.5 ml. (0.1 M) sodium carbonate solution. Record the resultant color. Any pink coloration appearing within 30 minutes is recorded as a positive reaction (presence of free phenolphthalein).

Chapter 15

SURVEILLANCE OF BACTERIAL INFECTION

15–1. Introduction. This chapter discusses briefly the organization and operation of an infection control unit, or infection committee, and the role of the bacteriology department in the control of hospital-acquired, or nosocomial, infections. The responsibility of the infection committee is to monitor the hospital environment and to prevent the spread of infection. Since infectious agents must be cultured and identified before they can be controlled, the part played by the bacteriology department is apparent. The bacteriology department should work very closely with other members of the infection committee to insure the best possible job of infection control.

15–2. Hospital-Acquired Infections:

a. **Definition.** Hospital-acquired infections are those infections acquired in the hospital (during hospitalization) whether from casual exposure or from practices peculiar to patient care. The infection may appear in the hospital or after the patient has been convalescing at home. Infections seen in the hospital are as a rule divided into three categories: (1) infections contracted prior to admission (prehospitalization), (2) infections contracted during hospitalization (hospital-acquired), and (3) post-hospitalization (convalescent) infections. It is sometimes difficult without good record keeping to determine what is and what is not a hospital-acquired infection. All committee efforts are toward a low incidence of hospital infections. Naturally, the smaller the number of infections acquired in the hospital, the better the hospital.

b. **Concerns of the Infection Committee:**

(1) The basic concern of the infection committee is to determine whether an infection which complicated treatment or recovery from an illness or wound was introduced into the patient's physical environment or physiological system during hospitalization in the present facility.

(2) The next consideration is a method of monitoring hospital infections. Some routine method or procedure is necessary to investigate and identify the bacteria involved in the inflammatory process and to determine whether the infection was acquired during the patient's hospitalization.

c. **Record-Keeping.** There is, therefore, a need for the laboratory to maintain records of each organism recovered until such time that it can be determined whether or not the infection was hospital acquired. Laboratory data on infection control should include the following information:

(1) The etiology of infection.

(2) Followups on patients.

(3) The presence of unusual microorganisms.

(4) Bacteriological, serological, and parasitological examination reports (if any).

(5) The results of routine checks of various areas being monitored.

15–3. Devising a System. In setting up a system for monitoring bacterial growth in surveillance areas, it is impractical to try to take all the cultures in one day. Sampling is best accomplished by preparing a schedule which requires only a few cultures a day, spread over a 30-day period. For example, cultures might be taken only on Monday and Wednesday by hospital personnel trained to augment the laboratory in the collection of samples around the hospital. Use of such a system prevents the technician from having to spend too much time away from the laboratory and the daily workload. Since paperwork is an integral part of a good surveillance program, forms of local design should be prepared to meet the needs of the facility engaging in monitoring of infection. Setting up a monitoring system involves the following basic steps:

a. Determining the locations and times for sample collections.

b. Culture of samples collected.

c. Reading out of cultures and preparing written reports.

d. Report of findings to concerned sections as well as to the Infection Committee.

e. Filing of collected data.

15–4. Role of Laboratory in Infection Control:

a. **Membership.** The laboratory should provide a member of the committee, usually the technician in charge of the department or the laboratory supervisor (or laboratory chief).

b. **Responsibilities.** Responsibilities of the laboratory are to:

(1) Maintain current data on antibiotic sensitivity patterns for Staphylococcus and gram-negative organisms of clinical importance.

(2) Protect laboratory personnel against infection and occupational hazards involved in the handling of infectious materials.

(3) Act as liaison with public health officials or reference laboratories for quality control.

(4) Report any microorganisms recovered from monitored areas.

(5) Provide checks on the sterilization systems used for bandages, linens, and medical instruments.

15–5. Reporting of Cultures and Other Data. In reporting the number of cultures taken to the infection committee, the number of positives should be noted. The report of beta streps, classified as "Group A" and "Other than Group "A", should be a routine part of the data provided to the Committee. Transfusions given during the month and any transfusion reactions should be reported. If a reaction to a transfusion takes place, blood cultures should be taken to insure that the urticara or rash is not due to the presence of a bacterial organism. Positive gonococcal (GC) cultures also are included in this report from data compiled by the environmental health section.

15–6. Composition of Infection Committee. Various members of the hospital staff are important to the establishment of a good hospital monitoring program. The committee should be composed of the pediatrician, surgeon, internist, nurse, and the hospital administrator, in addition to the laboratory representative. This committee should work very closely with the infection control nurse of the facility.

15–7. Conclusion. The information provided here concerning the control and surveillance of bacteria in a hospital environment should give some guidance to the technician working on a hospital monitoring program. A program for minitoring infection is only as good as its least effective committee member. Also, the control exercised by the infection committee is only as effective as the information provided by the bacteriology department permits. For more information on hospital infections, consult the following books:

a. **Nosocomial Infections.** Conference Proceedings, CDC, AHA, 1971.

b. **Hospital Infections: Causes and Prevention.** R.E.O. Williams, R. Blower et al, 2d ed, Yearbook, Medical Publications, 1966.

c. **Infection Control in the Hospital,** AHA 1970.

Chapter 16

ANAEROBIC BACTERIOLOGY

16-1. Introduction. Recent interest in anaerobic bacteriology has resulted in the development of better and more convenient methods for their isolation and identification. It was formerly thought that anaerobes were responsible only for tetanus, gas gangrene, spores, and bad smells, but it is now recognized that many anaerobes, particularly the toxigenic Clostridia and nonsporulating anaerobes, are pathogenic for humans. Many laboratories now attempt to isolate and identify the anaerobes as routinely as they do the aerobes. This chapter will consider procedures in anaerobic bacteriology and the media used to cultivate anaerobic bacteria.

16-2. Anaerobiosis. Some bacteria are only able to grow in the presence of oxygen; they are known as obligate aerobes. Others live and grow only in the complete, or almost complete, absence of oxygen; these are called obligate anaerobes. Those organisms which can live under either aerobic or anaerobic conditions are called facultative anaerobes. Anaerobic microorganisms are the predominant flora of humans and are found normally on mucous membranes and on other surfaces of the body. They are constantly present in the intestinal upper respiratory, and genitourinary tracts. Most are probably nonpathogenic for humans, but others are potential pathogens whenever they gain access to tissue that has been damaged through injury or surgical mishaps. In all types of infection, anaerobic bacteria are seen with aerobic and facultative bacteria. They are the predominant pathegens in many types of infections, for example, secondary pulmonary infection, liver abscess, wound infection following bowel surgery or trauma, endometritis, chronic otitis media, and bacteremia.

16-3. Anaerobic Recovery and Transport:

a. **Specimen Selection:**

(1) Avoid, if possible, those normal flora which can be recovered routinely from such areas as the skin, oropharynx, intestinal tract, and external genitalia.

(2) Choose instead specimens that seem likely to contain significant anaerobic organisms. Table 16-1 lists both specimens that routinely require anaerobic culture and those that do not.

(3) Possible sources of pathogenic anaerobes are listed below:

(a) Necrotic tissue or debridement from suspected gangrene.

(b) Material from infections close to mucous membrane.

Table 16-1. Specimens Requiring Anaerobic Culture and Those Not Routinely Anaerobic.

Not routinely anaerobic	Do culture anaerobically
throat, eye, ear	pus from abscesses
nose	urines (supropubic)
superficial wounds	pulmonary aspirations
urethra	uterine secretions (catherized)
vagina	
cervix	Do Use
rectum	1. Pre-reduced anaerobically sterilized media
bed sores (decubiti)	2. Freshly prepared media at all times (not older than 72 hours)
sputum	3. Gassed out collection tubes (tubes flushed out with oxygen-free CO_2)
bronchial secretions	
gastric content	
voided urine	
feces	

(c) Pus from any deep wound or aspirated material from an abscess, particularly if the material has a foul or fetid odor or contains "sulfur granules."

(d) Foods suspected of food poisoning or botulism.

(e) Fluids aspirated from an infected site, including peritoneal, pleural, and synovial fluids and blood specimens.

b. **Specimen Collection and Submission.** Some anaerobes cannot survive if exposed to the air. Therefore, specimens for anaerobic culture should be transported in special containers. Use collection methods which minimize contamination of specimens with aerobic bacteria and maintain the specimens in an anaerobic environment from the time of collection until delivery to the laboratory. Methods of collection and submission are discussed below and are summarized in table 16–2:

(1) *Needle and Syringe.* Aspirate a closed abscess through a decontaminated site. Expel any air from the syringe, leaving a solid column of material in the syringe. Shove the syringe into a sterile rubber stopper or cover it with a plastic sheath. The syringe and needle assembly containing the aspirated specimen may be taken directly to the laboratory if the specimen is processed promptly (the plastic sheath gradually admits air).

(2) *Transport Tubes.* Transport vials and tubes containing an oxygen free CO_2 atmosphere are available commercially. Specimens aspirated with a needle and syringe can be injected directly into the transport bottles. If necessary, a specimen tube can be opened in an upright position, the specimen or swab added, and the tube closed for transport to the laboratory. Since CO_2 is heavier than air, the CO_2 atmosphere is maintained in the transport tube.

(3) *Swab Samples.* Whenever possible, tissue samples or fluid aspirates should be collected rather than swab samples. The material on swabs should never be allowed to dry out. Specimens should be placed under anaerobic conditions immediately after collection for transport to the laboratory.

c. **Special Anaerobic Studies.** When routine aerobic and anaerobic cultures have not yielded a positive result, anaerobic cultures can be shipped to a reference laboratory or to the US

Table 16–2. Summary Chart (Specimens for Anaerobic Cultures).

SUMMARY CHART
(Specimens for Anaerobic Cultures)

Specimen	Recommended Method of Collection	Submit to Lab
1. Deep wounds and abscesses	Needle & syringe or swab	Needle & syringe anaport vial, swab in CO_2 gassed tube, or transport media
2. Pleural	Thoracentesis	Syringe or anaport vial
3. Pulmonary	Transtracheal aspiration	Syringe or anaport vial
4. Blood or bone	Needle & syringe	Aerobic & anaerobic Bactec vials
5. Surgical tissue		Anaport vial
6. Joint or other body fluids	Needle & syringe	Syringe, anaport vial, or aerobic and anaerobic Bactec
7. Urine	Suprapubic bladder aspiration	Syringe or anaport vial

Department of Health, Education, and Welfare, Center for Disease Control, Anaerobe Unit, Atlanta, Georgia, for identification. Notify the recipient laboratory in advance when and how the specimens are being shipped and when they should arrive.

16-4. Inoculating Media. Primary inoculation of specimens for anaerobic culturing requires several different types of media (see table 16-3). If the media is to be freshly prepared, it will require preparation on a daily basis, which may not be practical for many laboratories; however, alternatives are available in such instances. For example:

a. Use commercially prepared media.

b. Prepare plates and store them in an anaerobic jar at room temperature.

c. Media can be stored for not more than 72 hours in an air-tight cabinet under constant CO_2 pressure (for example, a CO_2 incubator). Media should never be refrigerated because lower temperature increases the absorption of atmospheric oxygen, which is toxic to obligate anaerobes.

16-5. Anaerobic Methods. Chapter 4 considers anaerobic methods in some detail and introduces several methods for providing anaerobic culture environment. The prereduced anaerobically sterilized (PRAS) method (Hungate), the anaerobic jar, and the anaerobic chamber or glove box are newer innovations in anaerobic bacteriology which increase the chances of recovering a clinically significant organism. In the absence of an anaerobic culture chamber, the Gas-Pak system appears to be the most practical method of providing anaerobic culture environment in the clinical laboratory (method discussed in chapter 4). The Gas-Pak is a very practical method that uses an anaerobic jar with a catalyst and a generator packet which liberates hydrogen (or nitrogen) plus 5 percent CO_2. An indicator strip is provided with the Gas-Pak. The strip is blue upon initial exposure but later changes to a white color, signifying the absorption of oxygen. The indicator may be freshly prepared by mixing equal amounts of glucose (6 percent), sodium hydroxide (1/10N), and aqueous methylene blue (0.5 percent). Boil a small amount (about 1 ml) until all oxygen has been driven from the solution. Place the tube in the loaded jar, and then seal the jar along with activated generator pack. If the solution in the tube remains colorless at the completion of incubation, anaerobiosis has been maintained throughout the growth period.

16-6. Primary Inoculation. A variety of liquid and solid media are available for primary inoculation of clinical specimens, most of which can be obtained from a commercial source. Commercial availability solves many of the preparation problems and also eliminates the need for maintaining storage of large numbers of dry media for preparation. In addition the need to maintain a stock of fresh media for dealing with anaerobic bacteria may be less of a problem especially if preparation cannot be made on a daily basis within the laboratory. However, in laboratories where small numbers of anaerobic cultures are processed, it may be expedient to prepare locally the media needed. Table 16-3 lists media for anaerobic cultures and their uses. Procedures for primary inoculations are as follows (inoculate immediately if possible):

a. **Fluid Specimens.** Use a capillary pipette to remove one or two drops of liquid or semisolid material from the bottom of the specimen. Place one drop on each plating medium and streak for isolation.

b. **Tissue (or Other Solid Specimens).** Mince material with sterile B-P blade, adding sufficient prereduced broth to emulsify the material, add sterile sand as necessary, and grind with a mortar and pestle. Treat as a liquid specimen from this point.

c. **Swabs.** Place swabs directly into liquid media and use another swab to streak plated media. If necessary, an inoculating suspension can be prepared by gently rubbing the specimen off the swab into approximately 2 ml of prereduced broth.

(1) Heat all liquid and semisolid media in boiling water bath for 10 minutes and cool prior to inoculation.

(2) Inoculate one tube each of thioglycollate and chopped meat glucose medium (for enrichment cultures), two blood agar plates (one aerobic and one anaerobic), selective media and egg yolk agar plate. Add 0.5 ml sterile rabbit serum to thioglycollate medium after the medium has been heated and cooled.

(3) Incubate cultures at 35°C to 37°C as follows:

(a) Incubate chopped meat and thioglycollate media in an anaerobe jar for 24 to 48 hours. Loosen caps to allow gases to escape.

(b) Incubate one BAP in a candle jar to determine aerobic flora.

(c) Incubate one BAP, one egg yolk

Table 16–3. Media and Their Uses.

Medium	Use
1. Chopped meat	Proteolysis - Digestion usually does not become apparent for several days
2. Fermentation base	Control for fermentation media
3. Fermentation media	Carbohydrate utilization
4. Thiogel	Gelatin liquefaction - Place cultures and control in beaker of cold water in refrigerator. Check for liquefaction as soon as the control is solid
5. Iron milk	Proteolysis
6. Indol-nitrite	Indol production - Extract with one dropper full of xylene. Add one dropper full of Ehrlich's reagent
7. Same as above	Nitrate reduction - Add one dropper full of solution A, 1/2 dropper full of solution B. If no color, add zinc dust
8. H_2S	H_2S production in lead acetate medium
9. Urea	Urease production - If phenol red indicator is reduced, add a few drops dilute phenol red to culture the last day of reading
10. Motility	Motility
11. Esculin	Esculin hydrolysis - Add one dropper full 1 percent ferric ammonium citrate. Read the immediate reaction
12. PYG (peptone-yeast extract-glucose)	Gas production
13. Infusion agar slant	Catalase. Expose slant air for 30 minutes before testing. Add about 1 ml of 3 percent H_2O_2 to growth on slant. Gas evolution indicates presence of catalase
14. Thioglycollate	Growth characteristics and control for 20 percent bile
15. 20 percent bile in thioglycollate	Growth in presence of 20 percent bile. Compare to growth in thioglycollate broth
16. Chopped meat-dextrose	Toxin testing on the Clostridia (only).

agar plate (EYA) and selective media in an anaerobic jar for a minimum of 48 hours, preferably 72 to 96 hours. If the presence of moisture is a problem place a sterile disk of filter paper in the top of the petri dish to absorb droplets.

(4) Following the 24-hour incubation period, inspect the enrichment cultures for growth. If no growth is apparent, reincubate cultures for another 24 hours. If growth is present, prepare a Gram stain for examination from the thioglycollate cultures. Remove some of the growth near the bottom of the tube using a long stem capillary pipette and inoculate the subculture media. Inoculate two BAP from growth in either the thioglycollate or the chopped-meat-glucose (CMG).

(5) Following incubation, examine all cultures using a hand lens or dissecting microscope to determine the presence of growth and hemolysis. Determine the number and types of colonies noted on plate. Pick individual colonies and transfer them to pre-reduced tubes of CMB and thioglycollate. Pre-reduce the media by boiling water bath for 10 minutes, cool prior to use. CMG is best for culturing the Clostridia while thioglycollate is good for the recovery of nonsporeforming anaerobes.

16-7. Cultural and Biochemical Characteristics:

a. Identification of anaerobic bacteria involves the determination of cellular morphology, colonial characteristics on BAP, along with the biochemical characteristics. (see tables 16-4 and 16-5). Clostridia can be tested for toxin production and the toxin can be identified by toxin neutralization procedures. These (toxin tests) are not routine laboratory procedures and therefore should be requested from reference facilities. Make gram stains to determine if a pure culture has been grown, and observe the size and shapes of organisms, and the location of spores (if present). Inoculate the basic differential media: chopped meat, fermentation base, glucose, mannitol, lactose, sucrose, maltose, salicin, thiogel, iron-milk, indolnitrite (for indol), indol nitrite (for nitrite), H_2S medium, motility, esculin, peptone yeast glucose (PYG) agar and an infusion agar slant. For gram positive spore formers add a urea tube to the battery, and for gram-positive non-sporeformers add glycerol, xylose, and arabinose. For gram-negative rods add the following sugar tubes: arabinose, xylose, rhaminose, trehalose. In the identification of the bacteria the technician should note colony characteristics and include the following information:

(1) Colony size.
(2) Age of the culture.
(3) Incubation temperature.
(4) Diameter.
(5) Pigmentation.
(6) Surface (glistening, dull, etc).
(7) Density (opaque, translucent, etc).
(8) Consistency (butyrous, viscid, membranous, brittle, etc).

b. Biochemical tests are routinely incubated up to 7 days after inoculation, but may be reported as early as 48 hours after initial incubation if reactions have reached completion. Otherwise the tubes are inspected daily and results are recorded from the first day to the final day of incubation. All tests on slow growing organisms should be held up to 3 weeks before discarding. The interpretation of other tests are as follows:

(1) Indol and nitrite are tested after 24-hours of browth and a good size sample can be used.

(2) Test for catalase on infusion agar and hydrolysis of esculin are normally made at 48 hours and the tubes are discarded.

(3) Thiogel may be incubated up to one month before it is reported as negative for liquefaction. The reaction is usually complete in about 7 days.

(4) Motility determination can be performed of SIM, but at times may not be possible due to gas production of the organism disrupting the media. In such cases, the motility can be checked by microscopic examination of a wet mount prepared from 6 to 18 hour old broth cultures grown in low-carbohydrate medium such as plain chopped meat medium (containing no glucose). For a summary of the purposes of the above mentioned differential media, consult table 16-3.

16-8. Identification of Anaerobic Bacteria. Two comprehensive charts have provided to aid in the identification of anaerobic bacteria based on biochemical testing, colonial morphology characteristics, cellular morphology, spore formation, and Gram stain (tables 16-4 and 16-5). Anaerobic bacteria will generally be found in one of the following groupings:

a. Clostridia.
b. Nonsporeforming gram positive bacilli:
(1) Actinomyces spp.
(2) Arachnia spp.
(3) Bifidobacterium spp.
(4) Eubacterium spp.

Table 16–4. Differentiation of Anaerobic Bacteria by Biochemical Tests.

DIFFERENTIATION OF ANAEROBIC BACTERIA BY BIOCHEMICAL TESTS
(Results read after 48 hours incubation under anaerobic conditions.)

Organism	IND	URE	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA	CEL	ESC	GLY	CEL	MNE	MLZ	RAF	SOR	RHA	INE	CAT
Clostridium perfringens	-	-	+	-	+	+	+	-	-	-	+	-	V		+	-	V	-	-	+	-
C. botulinu	-	-	+	-	-	+	+	-	-	-	+	-	+	-	+	-	-	+	-	-	-
C. ramosum	-	-	+	+	+	+	+	+	-	-	V	+	-	+	V	-	-	-	-	+	-
C. septicum	-	-	+	-	+	-	+	V	-	-	V	+	-	+	V	-	-	-	-	+	-
C. sordellii	+	+	+	-	V	-	V	-	-	-	V	V	-	-	V	-	-	-	-	-	-
C. sporogenes	-	-	V	-	-	-	V	-	-	-	+	+	-	-	-	-	-	-	-	-	-
C. sphenoides	+	-	+	+	+	+	+	V	V	+	-	+	±	+	+	-	+	-	+	-	-
C. innocuum	-	-	+	V	-	+	-	+	-	-	-	+	-	+	+	-	V	-	-	-	-
C. difermentans	+	-	+	-	-	-	+	-	-	-	+	+	-	-	-	+	-	-	-	-	-
C. butyricum	-	-	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+	-	-	+	-
C. paraputrificum	-	-	V	-	V	V	V	+	-	-	-	+	-	+	V	-	-	-	-	-	-
C. cadaveris	V	+	+	-	-	-	-	-	-	-	V	-	-	-	-	+	-	+	-	-	-
C. novyii A	-	+	+	-	-	±	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-
C. tertium	-	-	+	+	+	+	+	+	V	-	V	+	-	+	+	+	-	-	-	+	-
C. glycolicum	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Actinomyces israelli	-	-	+	+	V	+	+	+	+	V	V	+	-	+	-	+	+	+	+	-	-
A. odontolyticus	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	+	-
A. naeslundii	-	-	+	-	+	+	+	+	-	-	-	+	-	+	+	-	+	+	-	-	-
Bifidobacterium eriksonii	-	-	+	+	+	+	+	+	+	+	-	+	-	+	+	V	+	V	-	-	-
Eubacterium lentum	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-
E. limosum	-	-	+	+	-	-	-	-	-	-	-	±	-	+	+	-	-	-	-	-	+
Propionibacterium acnes	+	-	+	-	-	-	-	-	-	-	V	V	+	-	+	-	-	V	-	-	+
P. granulosum	-	-	+	+	-	+	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-
Bacteroides fragilis ssp. fragilis	-	-	+	-	+	+	+	-	+	-	-	+	-	V	+	±	-	-	-	+	-
B. fragilis ssp. thetaiotaomicron	+	-	+	-	+	+	+	V	+	-	-	+	-	±	+	-	+	-	-	-	-
B. fragilis ssp. vulgatus	-	-	+	-	+	+	+	-	+	V	-	V	-	±	+	-	+	-	-	V	-
B. fragilis ssp. distasonis	-	-	+	-	+	+	+	V	V	V	V	+	-	±	+	+	+	-	-	+	-
B. fragilis ssp. ovatus	+	-	+	+	+	+	-	+	V	V	-	+	-	+	+	V	+	-	-	+	-
B. melaninogenicus ssp. asaccharolyticus	±	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
B. melaninogenicus ssp. intermedius	+	-	+	-	-	V	V	-	-	-	+	-	±	-	V	-	±	-	-	+	-
B. oralis	-	-	+	-	+	+	+	-	-	-	+	+	-	+	+	-	+	-	-	-	-
B. biacutus	-	-	+	-	+	+	+	+	+	+	-	+	-	+	+	-	+	-	-	V	-
Fusobacterium mortiferum	-	-	+	-	+	+	+	+	-	-	V	+	V	V	+	-	-	-	-	-	-
F. necrophorum	+	-	+	-	-	-	±	-	-	-	-	±	-	-	-	-	-	-	-	-	-
F. nucleatum	+	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F. necrogenes	+	-	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-
Veillonella alcalescens	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	+
V. parvula	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-
Peptococcus asaccharolyticus	+	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-
Pc. magnus	-	-	V	-	-	-	-	-	-	-	V	-	-	-	±	-	-	-	-	-	-
Peptostreptoccus anaerobius	-	-	+	-	-	-	+	+	+	+	-	-	-	-	±	-	-	-	-	-	-
P. intermedius	-	-	+	+	+	+	+	+	+	±	-	+	-	+	+	±	±	-	-	+	-

Table 16-4. Continued.

INTERPRETATION OF RESULTS

TUBE	SUBSTRATE	TEST FOR	REACTIONS	
			POSITIVE	NEGATIVE
IND	Tryptophane	Indole	Red	Yellow
URE	Urea	Urease	Red	Yellow or orange
GLU	Glucose	Acid Production	Yellow-green	Purple
MAN	Mannitol	" "	"	"
LAC	Lactose	" "	"	"
SAC	Sucrose	" "	"	"
MAL	Maltose	" "	"	"
SAL	Salicin	" "	"	"
XYL	d (+) xylose	" "	"	"
ARA	l (+) arabinose	" "	"	"
GEL	Gelatin	Gelatinase	Dispersion of black particles	No dispersion of black particles
ESC	Esculin[1]	Esculinase[1]	Reddish-Brown[1,2]	Colorless or Black[1]
GLY	Glycerol	Acid Production	Yellow-green	Purple
CEL	Cellobiose	" "	"	"
MNE	Mannose	" "	"	"
MLZ	Melizitose	" "	"	"
RAF	Raffinose	" "	"	"
SOR	Sorbitol	" "	"	"
RHA	Rhamnose	" "	"	"
TRE	Trehalose	" "	"	"

[1]Doubtful reactions must be confirmed by long wave length UV. (366 nm).
Positive Reaction: No Fluorescens. Negative Reaction: Flourescens.

[2]Black deposit in bottom of tube = H_2S

* *

KEY FOR INTERPRETATION CHARTS

\+ Test result generally positive
\- Test result generally negative
+ Test result more often positive but negatives occur
\- Test result more often negative but positives occur
v Test result variable

MILK -

C Coagulation
G Gas produced
D Digestion
NC No coagulation
( ) Variable

GRAM REACTION -

\+ Gram-positive
\- Gram-negative
v Gram stain results may be either + or -

(5) Propionbacterium spp.

c. Nonsporeforming gram negative bacilli:
(1) Bacteroides spp.
(2) Fusobacterium spp.

d. Anaerobic cocci:
(1) Peptococcus spp.
(2) Peptostreptococcus spp.
(3) Veillonella spp.

16-9. Shipment of Anaerobic Cultures. The identification of anaerobes is often time consuming and beyond the scope of the smaller medical facility; anaerobic cultures should therefore be sent to a reference laboratory. In such cases anaerobic cultures can be shipped to the reference facility for identification in tubes of liquid or semisolid media. Plates or slant

Table 16–5. Other Characteristics Important in the Identification of Anaerobes.

OTHER CHARACTERISTICS IMPORTANT IN THE IDENTIFICATION OF ANAEROBES

Organism	Unique Colonial Morphology Characteristics	Gram Reaction	Cellular Morphology	Spores	Gelatin	Lipase	Lecithinase	Motility	Indole
C. perfringens	Double zone, Beta hemolysis	V	bacilli, rounded to flat ends	+[1]	CG		+		V
C. botulinum		+	bacilli, large	+	D or S	+		+	
C. ramosum		+	bacilli, pleomorphic, swellings in cells	+[1]	(C) (G)				
C. septicum	Irregular, rhizoid edge	+	bacilli, long, thin or lemon shaped	+	(C) (G)			+	+
C. sordelli		+	bacilli	+	NG		+	+	
C. sporogenes	raised center, flat rhizoid edge	+	bacilli	+	CD	+		+	V
C. sphenoides		V	bacilli	+	(C) (G)			+	V
C. tetani		+	bacilli	+	NG				
C. bifermentens		+	bacilli	+	D		+	+	
C. butyricum		+	bacilli	+	C			+	
C. paraputrificum		+	bacilli	+	C			+	+
C. cadaveris		+	bacilli	+	D			+	
C. novyi A	ground glass appearance	+	bacilli	+	C	+	+	+	
C. tertium	aerobic growth	+	bacilli	+	(C) (G)			+	+
C. bifermentans		+	bacilli	+	NG			+	
A. israelii	"molar-tooth"	+	bacilli, long filamentores; some branching	-	(C)	+			+
A. odontolyticus		+	bacilli	-	(C)				+
A. naeslundii		+	bacilli, long with short branches	-	NG				+
A. eriksonii		+	bacilli, pleomorphic	-	NG				
A. lentum		+	coccobacilli, pleomorphic, chains	-	NG				+
A. timosum		+	bacilli, pleomorphic, pairs & chains	-	NG				
A. acnes	white to pinkish	+	bacilli, club shaped	-	C				+
A. propionicum		+	bacilli, club shaped	-	C				
B. fragilis ssp. fragilis		-	bacilli, rounded ends	-	C	-	-	-	-
B. fragilis ssp. thetaiotaomicron		-	bacilli, rounded ends	-	C	-	-	-	-
B. fragilis ssp. ovatus		-	bacilli, rounded ends	-	(C)	-	-	-	-
B. fragilis ssp. distasonis		-	bacilli, rounded ends	-	C	-	-	-	-
B. fragilis ssp. vulgatus		-	bacilli, rounded ends	-	C	-	-	-	-
B. melaninogen ssp. oralis (haroldsii)	brown-black colonies (5+ days)	-	coccobacilli or elongated bacilli	-	CD	-	-	-	-
B. melaninogen ssp. intermedius	brown-black colonies (5+ days)	-	coccobacilli or elongated bacilli	-	CD	V	-	-	-
B. oralis	yellowish to translucent	-	bacilli	-	C	-	-	-	-
B. buccae		-	bacilli	-	C	-	-	-	+
B. morbiferum	"fried egg"	-	bacilli, pleomorphic, round swollen bodies	-	(C)	-	-	-	-
B. necrophorum	raised opaque center, translucent edge	-	bacilli, pleomorphic, round or tapered ends	-	NG	+	-	-	-
B. nucleatum	"bread crumbs"	-	bacilli, filamentores w/or w/o pointed ends	-	NG	-	-	-	-
B. necrogenes		-	bacilli	-	NG	-	-	-	-
M. alcalescens		-	cocci, short chains, irregular clumps	-	NG	-	-	-	+
M. parvula		-	cocci, pairs short chains or irregular clumps	-	NG	-	-	-	+
P. anaerobius		+	cocci, spherical	-	NG	-	-	-	-
P. asaccharolyticus		+	cocci, spherical	-	NG	-	-	-	-
P. intermedius		+	cocci, spherical or elongated	-	NG	-	-	-	+
P. intermedius		+	cocci, spherical or elongated	-	C	-	-	-	+

[1]spores often difficult to demonstrate.

media are not recommended for shipping cultures. Specimens should be in pure culture before shipping for identification. The media used to ship cultures should be carbohydrate free and should contain 0.3 to 1 percent agar, as in motility medium. It should be freshly prepared and inoculated 2 to 3 inches deep in screw-cap tubes. Clostridium cultures can be shipped in chopped meat media. Nonsporeformers can be shipped in thioglycollate medium. After the bacteria has been inoculated to the tube, a ¾ to 1-inch overlay of melted paraffin or 5 percent agar should be added to actively growing cultures in either semisolid or liquid media. The screw caps should be tightened and sealed by dipping the top of the sealed tube into the remaining melted paraffin or wrapped with parafilm strips. Care should be taken to ensure there is no leakage or breakage during shipment, if the facility is some distance away. See section on shipment of specimens in chapter 2 of this manual (paragraph 2-4).

16-10. Special Instructions:

a. Prereduced anaerobic transport containers should be issued by Microbiology Section to wards which would serve as distribution points for users from other wards.

b. Ward and clinical personnel should be responsible for maintaining a supply of anaerobic vials (anaport), anaerobic swabs (anaswab), and tubed anaerobic transport medium.

c. Anaerobic transporters should be used only for those specimens which are considered appropriate for anaerobic studies: namely, deep wounds and abscesses, transtracheal aspirates, suprapubic aspirates, joint and body fluids, and surgical tissue.

d. A specific storage area should be selected and appropriately marked. Keep prepared culture bottles and tubes at room temperature and transport media in the refrigerator. Expiration dates extend for 5 to 6 weeks. However, vials or tubes evidencing a blue color should not be used. Return to Microbiology for a fresh prereduced replacement.

e. Ward personnel should familiarize themselves with local directives. Any questions or problems relating to the anaerobic bacteriology program may be referred to the Chief, Microbiology Section.

BY ORDER OF THE SECRETARIES OF THE AIR FORCE AND THE ARMY

OFFICIAL

DAVID C. JONES, General, USAF
Chief of Staff

JAMES J. SHEPARD, Colonel, USAF
Director of Administration

OFFICIAL

BERNARD W. ROGERS
General, United States Army
Chief of Staff

PAUL T. SMITH
Major General, United States Army
The Adjutant General

SUMMARY OF REVISED, DELETED, OR ADDED MATERIAL

This manual revises and updates bacteriologic procedures; emphasizes the use of new reagents and procedures; and presents background information and procedures in a completely revised format. A chapter on clinical laboratory monitoring of nosocomial disease has been added, and nomenclature throughout the manual has been changed to conform with that in *Bergey's Manual of Determinative Bacteriology*, 8th edition. The chapter on bacteriology of dairy products has been deleted, and the chapters on bacteriology of water and food and on laboratory organization and management has been omitted, but the subjects are treated in other sections of the manual.

DISTRIBUTION:
Air Force: F
Army: Active Army, ARNG, USAR: To be distributed in accordance with DA Form 12-34B requirements for, Laboratory Procedures.

REAGENTS AND STAINS

Acetone-Alcohol (Decolorizer for Gram's Stain) (1)

Acetone	1 volume
Ethyl alcohol, 95 percent	1 volume

NOTE: Increasing the proportion of acetone to ethyl alcohol speeds the decolorizing process; reducing the proportion of acetone to ethyl alcohol slows the decolorizing process.

Actone-Alcohol, Ether (Decolorizer for Gram's Stain) (2)

Acetone	45 volumes
Ethanol	45 volumes
Menthanol	5 volumes
Ether	5 volumes

Mix the desired quantity in the above proportions and store in refrigerator until use.

Acid-Alcohol (Decolorizer for Acid-Fast Stain) (3)

Hydrochloric acid (HC1), concentrated	3 ml
Ethyl alcohol, 95 percent q.s. to	100 ml

Using a transfer pipette, place 3 ml of concentrated hydrochloric acid in a 100 ml volumetric flask and q.s. to 100 ml with 95 percent ethyl alcohol.

Alcohol, Formula for Dilution From 95 Percent Alcohol (4)

STATEMENT OF EQUATION:

Percent alcohol desired is to 95 percent alcohol as the number of ml of 95 percent alcohol to be used is to 100 m.

Example:

How much 95 percent alcohol should be q.s. to 100 ml to make up 100 ml of 85 percent alcohol?

85:95::×:100 or 8500 *divided* by 95 equals 89.4 ml of alcohol to be q.s. to 100 ml.

Example:

How much 95 percent alcohol should be q.s. to 100 ml to make up 100 ml of 70 percent alcohol?

70:95::×:100 or 700 *divided* by 95 equals 73.6 ml of alcohol to be q.s. to 100 ml.

Alpha-Naphthol Reagent (5)

Alpha-naphthol (1-naphthol)	5 g
Ethyl alcohol, absolute, q.s. to	100 ml

Dissolve the naphthol in less than 100 ml of absolute ethyl alcohol. Transfer this solution to a 100-ml volumetric flask and q.s. to 100 ml with absolute alcohol.

Alpha-Naphthylamine Reagent (6)

Alpha-naphthylamine	5 g
Acetic acid (5 N), 30 percent q.s. to	1 liter

Dissolve the alpha-naphthylamine in less than 1000 ml of 5N acetic acid. Transfer this solution to a 1-liter volumetric flask and q.s. to 1000 ml with 5 N acetic acid. Filter through washed absorbent cotton. An alternate method is to add 6 ml of dimethylalpha-naphthylamine to a 1-liter volumetric flask and q.s. to 1000 ml with 5 N acetic acid.

Aqueous Neutral Red Solution, 0.2 Percent (7)

Neutral red	0.2 g
Distilled water	100.0 ml

Dissolve powder in the distilled water.

Auramine "O" Stain (Morse) (8)

Auramine "O"	20 g
Glycerol	40 ml
Phenol	60 ml
Distilled water	900 ml

Place the dye in a 1000-ml Erlenmyer flask. Mix for 5 minutes on a magnetic stirrer. Thoroughly rinse the phenol-glycerol container with some of the water, adding the rinse water to the flask containing the dye. Add the balance of the water, and cover the flask with a paraffin film. Mix for 3 days on a magnetic stirrer; then store in a dark place for 3 to 4 days. Filter through a cellulose acetate filter (Millipore AP20) and store in a brown bottle.

Auramine-Rhodamine Stain (9)

Auramine "O"	1.50 g
Rhodamine B	0.75 g
Glycerol	75.00 ml
Phenol, liquefied	10.00 ml
Distilled water	50.00 ml

Place the dye in a 250-ml beaker, adding 25 ml of distilled water. Add the phenol to the solution and mix carefully. Now add the glycerol and the remaining water, mixing well. Filter the solution through glass wool to clear. The solution may be stored at room temperature, or placed in a refrigerator (temperature 4°C) where it will keep for several months.

Basic Fuchsin-Tannic Acid (Flagellar Stain) (10)

Basic fuchsin-tannic acid dye	1.7 g
Ethyl alcohol, 95 percent	35.0 ml
Distilled water	65.0 ml

Dissolve the dye in 35 ml of 95 percent ethyl alcohol. Transfer this solution to a 100-ml volu-

metric flask and q.s. to 100 ml with distilled water.

Borax Methylene Blue Counterstain (for Flagella Stain) (11)

Methylene blue	0.1 g
Borax	1.0 g
Distilled water, q.s. to	100.0 ml

Dissolve the methylene blue and borax in less than 100 ml of distilled water. Transfer this solution to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Buffered Dilution Water (pH 7.2) (12)

STOCK PHOSPHATE BUFFER SOLUTION (SOLUTION A)

Potassium dihydrogen phosphate (KH_2PO_4)	34 g
Distilled water, q.s. to	1 liter

Dissolve the potassium dihydrogen phosphate in 500 ml of distilled water. Adjust the pH to 7.2 with 1 N sodium hydroxide and q.s. to 1 liter with distilled water.

WORKING PHOSPHATE BUFFER SOLUTION (SOLUTION B)

Add 1.25 ml of stock buffer solution (Solution A) to 1 liter of distilled water. If sterile buffered dilution water is desired, pour the buffered dilution water into dilution bottles and sterilize by autoclave.

Carbol-Fuchsin (Strong) (13)

Basic fuchsin	10 g
Ethyl alcohol, 95 percent	100 ml
Phenol	5 g
Distilled water	100 ml

Dissolve the fuchsin in alcohol and store overnight at 37°C. Mix the phenol in water. Prepare a working stain by adding 10 ml of fuchsin solution to 100 ml of phenol solution. Filter.

Carbol-Fuchsin (Flagella Stain: Cesares-Gill) (14)

Basic fuchsin	10 g
Ethanol, 95 percent	100 ml
Phenol	5 g
Distilled water	100 ml

Dissolve the basic fuchsin in the alcohol. Store this solution overnight at 37°C. Mix the phenol in water and dissolve. Add the alcoholic fuchsin to the phenol solution. Before use, dilute the phenol-fuchsin solution 1:15 with distilled water (1 part to 14 parts water).

Carbol-Fuchsin Stain (for Modified Kinyoun Method of Acid-Fast Stain) (15)

Basic fuchsin	4 g
Ethyl alcohol, 95 percent	20 ml
Phenol, concentrated	8 ml
Distilled water	100 ml

Dissolve the basic fuchsin in 20 ml of 95 percent ethyl alcohol. While shaking the mixture, slowly add 100 ml of distilled water to the solution of basic fuchsin. Melt the phenol in a 56°C water bath and pipette 8 ml of phenol into the mixture.

Carbol-Fuchsin Stain (for Ziehl-Neelsen Method of Acid-Fast Stain) (16)

Basic fuchsin	1 g
Ethyl alcohol, 95 percent	10 ml
Phenol, 5 percent aqueous q.s. to	100 ml

Dissolve the basic fuchsin in 10 ml of 95 percent ethyl alcohol. Add 10 ml of basic fuchsin solution to a 100-ml volumetric flask and q.s. to 100 ml with 5 percent aqueous solution of phenol.

Copper Sulfate Solution (for Anthony Capsule Stain and/or Hiss Capsule Stain) (17)

Copper sulfate, C.P. ($CuSO_4$)	20 g
Distilled water, q.s. to	100 ml

Dissolve the copper sulfate in 50 to 75 ml of distilled water. Dissolving can be hastened by grinding the copper sulfate with mortar and pestle before weighing. Transfer the concentrated copper sulfate solution to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Cresol, 2 Percent (18)

Cresol, concentrated	20 ml
Distilled water, q.s. to	1000 ml

Transfer 20 ml of concentrated cresol (melt cresol by placing a bottle of the reagent in warm water) to a 1-liter graduate or volumetric flask. Dilute to the 1-liter mark with distilled water.

Crystal Violet Solution (for Anthony Capsule Stain and/or Hiss Capsule Stain) (19)

Crystal violet	1 g
Distilled water, q.s. to	100 ml

Dissolve the crystal violet in less than 100 ml of distilled water. Transfer this solution to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Crystal Violet (for Gram's Stain) (20)

SOLUTION A

Crystal violet	4 g
Ethyl alcohol, 95 percent	20 ml

SOLUTION B

Ammonium oxalate $(NH_4)_2C_2O_4.H_2O$	0.8 g
Distilled water	80.0 ml

Mix solutions A and B ordinarily in equal parts. Sometimes this procedure may cause some organisms, such as the gonococcus, to retain the basic dye and resist decolorization. To avoid this, solution A may be diluted as much as 10 times. Then solution A is added in equal parts to solution B.

Cyanogen Bormide, 10 Percent (21)

Cyanogen bromide (CNBr)	10 g
Distilled water, q.s. to	100 ml

Dissolve the cyanogen bromide in less than 100 ml of distilled water. Transfer the solution to a 100-ml volumetric flask and q.s. to 100 ml with distilled water. CAUTION: This solution must be prepared under a fume hood. If this solution must be transferred by pipette, use a rubber-bulb pipette.

Ferric Chloride, 10 Percent (22)

Ferric chloride ($FeCl_3$)	10 g
Distilled water, q.s. to	100 ml

Dissolve the ferric chloride in less than 100 ml of distilled water. Transfer this solution to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Ferric Chloride, 12 Percent (23)

Ferric chloride ($FeCl_3$)	12 g
Hydrochloric acid, 2 percent aqueous q.s. to	100 ml

Dissolve the ferric chloride in less than 100 ml of the 2 percent hydrochloric acid. Transfer this solution to a 100-ml volumetric flask and q.s. to 100 ml with 2 percent hydrochloric acid.

Flagella Stain Fixative (24)

Ethanol, absolute	60 ml
Chloroform	30 ml
Formaldehyde	10 ml

Mix the ingredients in the above order and store in a tightly capped bottle.

Formaldehyde, 4 Percent (25)

Formalin (formaldehyde, 40 percent)	10 ml
Distilled water, q.s. to	100 ml

Transfer 10 ml of formalin to a 100 ml volumetric flask and q.s. to 100 ml with distilled water.

Hydrochloric Acid, 50 Percent (26)

Hydrochloric acid (HCl), concentrated, R. G.	50 ml
Distilled water, q.s. to	100 ml

Place the concentrated hydrochloric acid in a 100 ml volumetric flask and q.s. to 100 ml with distilled water.

Glucose Solution, 50 Percent (27)

Carbohydrate	50 g
Distilled water	100 ml

Dissolve and sterilize at 121°C for 15 minutes.

Hydrochloric Acid 2.5 N (28)

Hydrochloric acid (HCl), concentrated, R. G.	21 ml
Distilled water, q.s. to	100 ml

Place 21 ml of concentrated hydrochloric acid in a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Hydrochloric Acid, 2N (29)

Hydrochloric acid (HCl), concentrated, R. G.	17 ml
Distilled water, q.s. to	100 ml

Place 17 ml of concentrated hydrochloric acid in a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Hydrochloric Acid, 3 Percent (30)

Hydrochloric acid (HCl), concentrated	3 ml
Distilled water, q.s. to	100 ml

Using a transfer pipette, place 3 ml of concentrated hydrochloric acid in a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Hydrochloric Acid, 2 Percent (31)

Hydrochloric acid (HCl), concentrated	2 ml
Distilled water, q.s. to	100 ml

Using a transfer pipette, place 2 ml of concentrated hydrochloric acid in a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Hydrochloric Acid, 0.2 N (N/5) for Lancefield Precipitin Test (32)

Hydrochloric acid (HCl), concentrated	1 ml
Physiological saline	59 ml

Using a transfer pipette, place 1 ml of concentrated (12N) hydrochloric acid in a flask and add 59 ml of physiological saline.

Iodine Solution (for Gram's Stain) (33)

Iodine (I_2)	1 g
Potassium iodide (KI)	2 g
Sodium bicarbonate ($NaHCO_3$), 5 percent	60 ml
Distilled water	240 ml

Grind the iodine and potassium iodide in a mortar. Dissolve the ground reagents in 5 ml of distilled water and q.s. to 240 ml with distilled water. Add 60 ml of the 5 percent aqueous

sodium bicarbonate (5 g sodium bicarbonate q.s. to 100 ml with distilled water). When a color loss of the Gram's iodine is noted, prepare a fresh solution. Gram's iodine solution will remain stable for a longer period of time when stored in a dark bottle.

Indicator Solutions (34)

With the exception of phenol red indicator solution for concentration technique, 0.4 percent, all other indicator solutions are prepared by dissolving the indicator in 50 ml of 95 percent ethyl alcohol. The resulting solution is then transferred to a 100-ml volumetric flask and diluted to the mark with distilled water.

BROMCRESOL PURPLE INDICATOR SOLUTION, 0.2 percent (34a)

Bromcresol purple	0.2 g
Ethyl alcohol, 95 percent	50.0 ml
Distilled water	50.0 ml

NOTE: Sensitive pH range Yellow 5.2–6.8 Purple

Add 5 ml of the 0.2 percent solution of bromcresol purple indicator to each liter of medium requiring this indicator.

BROMTHYMOL BLUE INDICATOR SOLUTION, 0.4 percent (34b)

Bromthymol blue	0.04 g
Ethyl alcohol, 95 percent	50.00 ml
Distilled water	50.00 ml
NOTE: Sensitive pH range	Yellow 6.0–7.6 Blue
Sodium hydroxide, N/20	25.0 ml

Disolve the phenol red in 25 ml of N/20 sodium hydroxide and store the solution in a dark bottle.

PHENOL RED INDICATOR, 0.01 percent (34f)

Phenol red	0.01 g
Ethyl alcohol, 95 percent	50.00 ml
Distilled water	50.00 ml
NOTE: Sensitive pH range	Yellow 6.8–8.4 Red

THYMOL BLUE INDICATOR, 0.01 percent (34g)

Thymol blue	0.01 g
Ethyl alcohol, 95 percent	50.00 ml
Distilled water	50.00 ml

NOTE: Sensitive pH range (acid) Red 1.2–2.8 Yellow

Kovac's Reagent (35a)

Paradimethylaminobenzaldehyde, C.P.	5 g
Amyl or butyl alcohol	75 ml

Substitute 0.5 g for the 0.04 g of bromthymol blue indicator when preparing the solution of bromthymol blue for addition to a medium.

METHYL RED INDICATOR SOLUTION, 0.02 percent (34c)

Methyl red	0.02 g
Ethyl alcohol, 95 percent	50.00 ml
Distilled water	50.00 ml
NOTE: Sensitive pH range	Red 4.4–6.0 Yellow

PHENOL RED INDICATOR SOLUTION (FOR FERMENTATION MEDIA, 0.2 percent) (34d)

Phenol red	0.2 g
Ethyl alcohol, 95 percent	50.0 ml
Distilled water	50.0 ml
NOTE: Sensitive pH range	Yellow 6.8–8.4 Red

Add 10 ml of the 0.2 percent solution of phenol red indicator to each liter of medium requiring this indicator.

PHENOL RED INDICATOR SOLUTION FOR CONCENTRATION TECHNIQUE, 0.4 percent (34e)

Phenol red	0.1 g
Hydrochloric acid, (HCl) concentrated	25 ml

Dissolve the reagent in 75 ml of the alcohol. Transfer the solution to a 100-ml volumetric flask and q.s. to 100 ml with concentrated hydrochloric acid.

Kovac's Reagent (35b)

Pure amyl or isoamyl alcohol	150 ml
Paradimethylaminobenzaldehyde	10 g
Hydrochloric acid, (HCl) concentrated	50 ml

Dissolve the aldehyde in the alcohol and add the acid slowly. Prepare in small quantities and store in the refrigerator when not in use. Inoculate tryptophane broth and incubate for 48 hours at 35°C to 36°C. Add 5 drops of Kovac's reagent. A deep red color indicates the presence of indole. (Tryptophane medium should be obtained from BBL or Difco Lab.)

Lactic Acid, 10 Percent (36)

Lactic acid (2-hydroxypropanoic acid)	10 ml
Distilled water, q.s.	100 ml

Transfer 10 ml of concentrated lactic acid to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Lead Acetate, 10 Percent (37)

Lead acetate, $Pb(C_2H_3O_2)_2 \cdot 3H_2O$	10 g
Distilled water, q.s. to	100 ml

Dissolve the lead acetate in less than 100 ml of distilled water. Transfer the solution to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Lead Acetate Paper (38)

Cut filter paper into strips 5 to 10 mm wide and 50 to 60 mm long. Immerse the strips in a hot, saturate, aqueous solution of lead acetate. Drain and air-dry. Sterilize in a suitable container at 121°C for 15 minutes.

Levaditi Stain (39)

Silver nitrate solution, 2 percent	2.0 g
Distilled water	100.0 ml
Pyrogallic acid	4.0 g
Formaline (37 to 40 percent formaldehyde)	5.0 ml
Distilled water	100.0 ml

Wash block of fixed, unembedded tissue in tap water for several minutes. Place block in 95 percent alcohol for 24 hours. Place block in distilled water until the tissue sinks. Place in freshly prepared 2 percent silver nitrate solution at 37°C in the dark for 4 days, changing the solution daily. Wash in distilled water. Place in the reducing solution (Pyrogallic acid, formalin, and distilled water) for 48 hours. Wash in several changes of distilled water. Carry through the alcohol baths, embed in the regular manner, and section. Deparaffinize the sections with xylene and mount. Results: Spirochetes will be black and the remaining tissue, yellow and brown.

The Levaditi staining method is applied to blocks of tissue rather than to sections of tissue. Preparations of this nature often harbor numerous organisms, making their identification easier.

Löffler's Methylene Blue Stain (40)

Methylene blue	0.3 g
Ethyl alcohol, 95 percent	30.0 ml
Potassium hydroxide, 1:10,000 aqueous solution, q.s. to	100.0 ml

Dissolve the methylene blue in 30 ml of 95 percent ethyl alcohol. Transfer the concentrated methylene blue solution to a 100-ml volumetric flask and q.s. to 100 ml with a 1:10,000 aqueous solution of potassium hydroxide. This dilution of potassium hydroxide can be approximated by adding two drops of a 10-percent solution of potassium hydroxide to the 100-ml volume after q.s. with distilled water.

Malachite Green Solution (for Wirtz-Conklin Spore Stain) (41)

Malachite green	5 g
Distilled water, q.s. to	100 ml

Prepare a 5 percent aqueous solution of malachite green by dissolving the dye in a small amount of water. Transfer the concentrated solution of malachite green to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Mayer's Egg Albumin Fixative (42)

Egg whites	1 volume
Glycerin	1 volume

Mix the glycerin with the egg whites. Filter this mixture through sterile gauze into a flask and store at room temperature. Periodically, add a few crystals of thymol to maintain sterility of the mixture.

Mercuric Chloride Solution, 0.1 Percent (43)

Mercuric chloride ($HgCl_2$)	0.1 g
Distilled water, q.s. to	100.0 ml

Dissolve the mercuric chloride in a small amount of distilled water. Transfer the solution to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Metachromatic Stain (44)

Toluidine blue	0.15 g
Malachite green	0.20 g
Ethyl alcohol	2.00 ml
Glacial acetic acid	1.00 ml
Distilled water	100.00 ml

Mix the dyes and dissolve in the 2 ml of alcohol. Mix the acetic acid in the water. Combine the alcoholic stain solution and the acidified water. Mix well and let stand for 24 hours. Filter before use.

Methylene Blue Counterstain (45)

Methylene blue	0.3 g
Distilled water	100.0 ml

Dissolve the dye in a small portion of the water before adding the remainder.

Methyl Alcohol, 50 Percent (46)

Methyl alcohol, absolute	50 ml
Distilled water, q.s. to	100 ml

Place 50 ml of absolute methyl alcohol in a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Mordant (Flagella Stain: Cesares-Gill) (47)

Tennic acid	20 g
Aluminum chloride ($AlCl_3 \cdot 6H_2O$)	36 g
Zinc Chloride ($ZnCl_2$)	20 g
Basic fuchsin	3 g
Ethyl alcohol, 60 percent	80 ml

Triturate the solid ingredients together in a mortar. Add the alcohol and continue to grind until dissolved. Before use, add one volume of mordant to three volumes of distilled water. Mix well. In use, the mixture is filtered directly onto the slides.

Mordant (Metachromatic Stain) (48)

Iodine, crystals	2 g
Potassium iodide (KI)	3 g
Distilled water	200 ml

Mix the dry reagent and add 5 to 10 ml of the water to dissolve the solids. Add the balance of the water; mix by stirring. Store in a dark glass-stoppered bottle.

Mordant (Muir Capsule Stain) (49)

Mercuric chloride ($HgCl_2$) saturated solution	20 ml
Potassium alum, saturated solution	50 ml
Tannic acid, 10 aqueous	20 ml

Saturated solutions of mercuric chloride and potassium alum may be prepared by placing crystals of each in separate 25 ml Erlenmyer flasks until they no longer go into solution. The 10-percent tannic acid solution is a W/V solution (10 grams/100 ml solution). The mordant is prepared in the above proportions.

Ninhydrin Reagent (50)

Ninhydrin (1, 2, 3-triketohydrindene)	0.1 g
Chloroform, q.s. to	100.0 ml

Dissolve the ninhydrin reagent in less than 100 ml of chloroform. Transfer this solution to a 100-ml volumetric flask and q.s. to 100 ml with chloroform.

Oxidase Reagent (for Neisseria spp) (51)

Dimethylparaphenylenediamine hydrochloride	0.1 g
Distilled water	10.0 ml

Add the dye to the distilled water and observe for a purple color which should form in 10 to 20 minutes. The presence of the purple color indicates that the reagent is ready for use. This reagent begins to lose its activity in 1 to 2 hours after the solution has been prepared.

NOTE: Tetramethylparaphenylenediamine dihydrochloride may be substituted for dimethylparaphenylenediamine hydrochloride.

Peptone Water, 1 Percent (52)

Peptone	10 g
Sodium chloride (NaCl)	5 g
Distilled water, q.s. to	1000 ml

Dissolve the peptone and sodium chloride in less than 1 liter of distilled water. Transfer this solution to a 1000-ml volumetric flask and q.s. to 1000 ml with distilled water. Adjust the pH to 7.6 with 1 N sodium hydroxide. Tube and sterilize in the autoclave.

Phenol, 5 Percent (53)

Phenol, concentrated	50 ml
Distilled water, q.s. to	1 liter

Transfer 50 ml of concentrated phenol (melt phenol by placing a bottle of the reagent in warm water) to a 1-liter graduate or volumetric flask. Dilute to the 1-liter mark with distilled water.

Phosphate Buffer Solution (pH 7) (54)

Potassium dihydrogen phosphate (KH_2PO_4), anhydrous	1361 g
Distilled water, q.s. to	1000 ml

Dissolve the salts in a small amount of distilled water. Quantitatively transfer this concentrated solution to a 1-liter volumetric flask and q.s. to 1000 ml with distilled water. Use distilled water which has been boiled and cooled.

Physiological (Isotonic) Salt Solution (55)

Sodium chloride (NaCl)	8.7 g
Distilled water, q.s. to	100 ml

Dissolve the sodium chloride in approximately 100 ml of distilled water. Transfer the salt solution to a 1000-ml volumetric flask and q.s. to 1000 ml with distilled water. If sterile saline is desired, pour the physiological saline into flasks, plug, and sterilize in an autoclave.

Potassium Hydroxide, 40 Percent (56)

Potassium hydroxide (KOH)	40 g
Distilled water, q.s. to	100 ml

Rapidly weigh the potassium hydroxide (because of its hygroscopic nature) and dissolve in less than 100 ml of distilled water in a beaker. The beaker may be placed in the sink with cold water circulating around it to control temperature. Cool and transfer the solution of potassium hydroxide to a 100-ml volumetric flask and q.s. to 100 ml with distilled water. Store this solution in either a polyethylene or paraffin-lined glass reagent bottle equipped with a rubber stopper.

Potassium Permanganate, Counterstain (57)

$KMnO_4$	0.5 g
Distilled water	100.0 ml

Dissolve 0.5 g $KMnO_4$ in a 100-ml Erlenmyer flask, using about 50 ml of water and q.s. to 100 ml mark. Mix solution by gentle inversion of the flask.

Pyrogallic Acid, 40 Percent (58)

Pyrogallic acid	40 g
Distilled water, q.s. to	100 ml

Dissolve the pyrogallic acid in less than 100 ml of distilled water. Transfer this solution to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Ringer's Solution (59)

Sodium chloride	7.00 g
Potassium chloride	0.25 g
Calcium chloride (anhydrous salt)	0.30 g

To approximately 400 ml distilled water in a liter volumetric flask, dissolve the above reagents. Fill flask to mark and mix by inversion, store in a plastic bottle in refrigerated area. Before use, allow the solution to come to room temperature.

Safranin Counterstain (for Gram's Method) (60)

Safranin	0.5 g
Distilled water, q.s. to	100.0 g

Dissolve the safranin in a small amount of distilled water. Transfer this aqueous safranin solution to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Safranin Solution (for Wirtz-Conklin Spore Stain) (61)

Safranin	0.5 g
Distilled water	100.0 ml

Prepare a 0.5-percent aqueous solution of safranin by dissolving the safranin in a small amount of water. Transfer the concentrated solution of safranin to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Sodium Barbital 1 Percent (62)

Sodium barbital	1 g
Distilled water	100 ml

Dissolve the crystals in the water and store until time to be used.

Sodium Carbonate, 20 Percent (63)

Sodium carbonate (Na_2CO_3) C.P.	20 g
Distilled water, q.s. to	100 ml

Weigh the sodium carbonate and transfer it to a 100-ml volumetric flask. Dissolve and dilute to the mark with distilled water.

Sodium Chloride, 5 Percent (64)

Sodium chloride	5 g
Distilled water	100 ml

Dissolve salts in the distilled water and store for later use.

Sodium Citrate, 2 Percent (65)

Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	2 g
Distilled water	100 ml

Dissolve the sodium citrate in less than 100 ml of distilled water. Transfer the solution to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Sodium Desoxycholate Solution, 2 Percent (for Bile Solubility Test (66)

Sodium desoxycholate	2 g
Distilled water, q.s. to	100 ml

Dissolve the sodium desoxycholate in a small amount of water. Transfer the concentrated solution of desoxycholate to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Sodium Hydroxide, 10 N or 40 Percent (67)

Sodium hydroxide (NaOH), carbonate free, R.G.	40 g
Distilled water, q.s. to	100 ml

Rapidly weigh the sodium hydroxide (because of its hygroscopic nature) and dissolve it in less than 100 ml of distilled water in a beaker. The beaker may be placed in the sink with cold water circulating around it to control temperature. Cool and transfer the solution of sodium hydroxide to a 100-ml volumetric flask and q.s. to 100 ml with distilled water. Store this solution in either a polyethylene or paraffin-lined glass reagent bottle. If a paraffin-lined glass reagent bottle is used, it is advisable to use a rubber stopper to prevent the sticking of a glass stopper through the leaching action of sodium hydroxide.

Sodium Hydroxide, 4 N (68)

Sodium hydroxide, 10 N	20 ml
Distilled water, q.s. to	50 ml

Using a transfer pipette, place 20 ml of 10 N sodium hydroxide in a 50-ml volumetric flask and q.s. to 50 ml with distilled water.

Sodium Hydroxide, 1.0 N or 4 Percent (69)

Sodium hydroxide, 10 N	10 ml
Distilled water, q.s. to	100 ml

Using a pipette, transfer 10 ml of 10 N sodium hydroxide to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Sodium Hydroxide, 0.2 N (N/5), Buffered (70)

Sodium hydroxide, 10 N	1.0 ml
Distilled water, q.s. to	50.0 ml
Sodium dihydrogen phosphate (NaH_2PO_4), anhydrous	0.5 g

Using a pipette, transfer 1 ml of 10 N sodium hydroxide to a 50-ml volumetric flask and q.s. to 50 ml with distilled water. Add 0.5 g of sodium acid phosphate to each 50 ml of N/5 sodium hydroxide to provide N/5 sodium hydroxide in M/15 phosphate buffer solution for a resulting pH of 7.

Sodium Hydroxide, 0.1 N (71)

Sodium hydroxide, 10 N	1 ml

Distilled water, q.s. to ..... 100 ml

Using a pipette, transfer 1 ml of 10 N sodium hydroxide to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Sodium Hydroxide, N/20 (72)

Sodium hydroxide, 10 N ..... 0.5 ml
Distilled water, q.s. to ..... 100.0 ml

Transfer 0.5 ml of 10 N sodium hydroxide to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Sodium Thiosulfate Dechlorinating Solution (73)

Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) ..... 10 g
Distilled water, q.s. to ..... 100 ml

Dissolve the sodium thiosulfate in less than 100 ml of distilled water. Transfer this solution to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Sorensen's pH Buffer (74)

Solution A: Dissolve 9.073 g-KH_2PO_4 of previously dried (110°) anhydrous salt in 500 ml of distilled water mix and q.s. to 1000 ml.

Solution B:

Add pH	Solution A	Solution B
5.3	0.25 ml	9.75 ml
5.6	0.5 ml	9.5 ml
5.9	1.0 ml	9.0 ml
6.2	2.0 ml	8.0 ml
6.5	3.0 ml	7.0 ml
6.6	4.0 ml	6.0 ml
6.8	5.0 ml	5.0 ml
6.9	6.0 ml	4.0 ml
7.2	7.0 ml	3.0 ml
7.4	8.0 ml	2.0 ml
7.8	9.0 ml	1.0 ml
8.0	9.5 ml	0.5 ml

Sucrose (stock solution) (75)

Sucrose ..... 50 g
Distilled water ..... 50 ml

Add the sucrose to the water and mix. This results in a 50 percent stock solution of sucrose. Autoclave the solution at 15 pounds pressure (121°C) for 15 minutes and store in the refrigerator for future use.

Sulfanilic Acid Reagent (76)

Sulfanilic acid (p-aminobenzene-sulfonic acid) ..... 8 g
Acetic acid (5 N)m 30 percent q.s. to 1000 ml

Dissolve the sulfanilic acid in less than 1000 ml of 5 N acetic acid. Transfer this solution to a 1-liter volumetric flask and q.s. to 1000 ml with 5 N acetic acid.

Trisodium Phosphate, 23 Percent (77)

Trisodium phosphate ..... 23 g
Distilled water ..... 100 ml

Dissolve in water and store in a dark brown bottle.

L-Tryptophan, 0.2 Percent (78)

L-tryptophan ..... 0.2 g
Distilled water, q.s. to ..... 100.0 ml

Dissolve the tryptophan in less than 100 ml of distilled water. Transfer this solution to a 100-ml volumetric flask and q.s. to 100 ml with distilled water.

Tween-Peroxide Mixture (79)

Tween 80,* 10 percent ..... 1 volume
Hydrogen peroxide (Superoxol), 30 percent ..... 1 volume

*A polyoxyethylene derivative of sorbitan monocleate.

Prepare a 10-percent solution of Tween 80 by placing 1 ml of the concentrated Tween 80 in a 10-ml volumetric flask and diluting to the 10-ml mark with distilled water. To one volume of the 10-percent Tween 80, add an equal volume of 30-percent hydrogen peroxide (Superoxol).

Voges-Proskauer Reagents (80)

Alpha-naphthol (5 percent) in absolute ethyl alcohol.

Potassium hydroxide (40 percent) containing 0.3 percent creatine.

Wirtz-Conklin Stain (81)

Flood the entire slide with 5-percent aqueous malachite green. Steam for 3 to 6 minutes and rinse under running tap water. Counterstain with 0.5-percent aqueous safranine for 30 seconds. Spores are seen as green spherules in red-stained rods or with red-stained debris.

MEDIA*

In chapter 4, a general discussion of the preparation and use of bacteriological media is presented. In this attachment, specific formulations are described for some media cited in the text. Most media are available commercially in the dehydrated form. Since dehydrated products have been economically used with considerable success in many laboratories, it is recommended that commercial media be procured and prepared in accordance with the manufacturer's instructions.

For additional references with respect to preparation of bacteriological culture media, various directives and manuals distributed by Difco (Digestive Ferments Company) and BBL (Baltimore Biological Laboratories) may be consulted.

Amies Transport Medium

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Calcium chloride	0.1 g
Magnesium chloride	0.1 g
Monopotassium phosphate	0.2 g
Disodium phosphate	1.5 g
Sodium thioglycolate	1.0 g
Charcoal, finely powdered	10.0 g
Agar	3.6 g
Distilled water	1.0 g

Mix and heat gently to dissolve soluble ingredients. Dispense in small, screw-capped vials filled within 5 mm of the top. Tighten caps and sterilize at 121°C for 15 minutes. Invert vials just before gelation to suspend the charcoal evenly. Retighten caps if necessary. Use sterile nontoxic swabs or boil swabs in Sorensen phosphate buffer 0.067 M, pH 7.4, and dry before use. After a swab has been dipped into a specimen, insert it deep into the medium of a vial. Cut off the stick and replace the cap tightly.

Andrade's Indicator

Acid fuchsin	0.5 g
Sodium hydroxide (1.0N)	16.0 ml
Distilled water	100.0 ml

The fuchsin is dissolved in the distilled water and the sodium hydroxide is added. If, after several hours, the fuchsin is not sufficiently decolorized, add an additional 1 or 2 ml of alkali (NaOH). The dye content of different sample amounts of alkali which should be used with any particular sample usually is specified on the label. The indicator improves with aging. The indicator is used in amounts of 10 ml per liter of medium.

Baird-Parker Medium (Egg Yolk Pyruvate Tellurite Glycine Agar)

Pancreatic digest of casein USP	10 g
Beef extract	5 g
Yeast extract	1 g
Sodium pyruvate	10 g
Glycine	12 g
Lithium chloride	5 g
Agar, dried	17 g
or, not dried	20 g
Distilled water (pH 6.8)	1 liter

Autoclave at 121°C for 15 minutes. Cool to about 50°C and add 50 ml of egg yolk emulsion* and 10 ml of sterile 1 percent (or 3 ml of 3.5 percent) potassium tellurite solution. Mix well and pour into plates.

Blood Agar Base (Heart Infusion Agar)

Beef heart muscle, infusion from	375 g
Tryptose of thiotone peptic digest of animal tissue USP	10 g
Sodium chloride	5 g
Agar	15 g
Distilled water (pH 7.3 or pH 6.8)	1 liter

Autoclave at 121°C for 15 minutes. Cool to 50°C and add 5 percent sterile defibrinated rabbit blood, if desired.

Blood Agar Plates For Streaking

Infusion agar or trypticase soy agar	500 ml
Sterile blood (defibrinated)	25 ml

Prepare 500 ml of infusion agar or trypticase soy agar. Cool the sterilized agar to 48°C by placing the flask in a 48°C water bath. Add the sterile blood and rotate the flask gently until the blood is thoroughly mixed. Pour the agar in 10 to 12 ml amounts into sterile Petri dishes, using sterile technique throughout. When the agar has solidified, invert the plates, incubate overnight at 37°C to test for sterility. Store the blood plates in the refrigerator until needed.

*Commercially available.

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Blood agar plates may be refrigerated for about 10 days and still remain satisfactory for use.

The formation of surface air bubbles on blood agar must be avoided when pouring the plates. This may be accomplished by pouring the blood agar base through a layer of sterile cheese cloth.

Human, rabbit, sheep, or horse blood are used in preparing blood agar. Sheep blood is preferred.

Blood Agar Pour Plates

A number of standard, cotton-plugged culture tubes containing 12 ml of infusion agar or trypticase soy agar may be prepared and maintained under refrigeration. These are ideal for use in making pour plates as outlined below.

Heat the number of tubes required in a boiling water bath until the agar is completely melted. Cool the medium to 48°C by placing the tubes in a 48°C water bath. With a sterile pipette, aseptically add 0.7 to 1.0 ml of sterile, defibrinated blood to each tube. Quickly inoculate the fluid blood agar with the specimen. Thoroughly mix the blood, inoculum, and culture media by twirling the tube. Dispense to a sterile Petri dish and allow to solidify. Invubate at the proper temperature.

Bismuth Sulfite Agar, Modified

Base

Beef extract	5 g
Peptone: Thiotone *or* Thiopeptone *or* Polypeptone	10 g
Dextrose	5 g
Sodium chloride	5 g
Agar	25 g
Distilled water	1 liter

Autocalave at 121°C for 15 minutes. Cool to about 50°C and add 70 ml of the following:

Bismuth Sulfite Additive

First dissolve 200 g of anhydrous sodium sulfite in 1 liter of distilled water. Next, dissolve 50 g of bismuth ammonium citrate in 500 ml of distilled water. Add 1 ml of concentrated ammonium hydroxide. Allow to stand until clear, adding several more millilters of ammonium hydroxide, if necessary. The two solutions are mixed together at this point. Add and mix 100 g of anhydrous dibasic sodium phosphate. Next, dissolve 10 g of ferric ammonium sulfate in 100 ml of distilled water, mixing with the solution. Heat the combined solution at 100°C for 2 to 3 minutes. Stopper the container and store in a dark place at room temperature. (Do not refrigerate or autoclave this solution.)

Shake the mixture of base and bismuth sulfite solution thoroughly. Add 4 ml of 1 percent aqueous solution of brilliant green. Cool to 40 to 50°C and pour into plates.

Bordet Gengou Agar, Modified

Without Peptone

Potatoes, washed, peeled, sliced	125.0 g
Glycerol	10.0 ml
Sodium chloride	5.6 g
Agar	22.5 g
Distilled water	1.0 liter

Place potatoes in loose gauze bag. Submerge in mixture of glycerol and half of the water. Boil until potatoes are soft. Strain through gauze and allow fluid to stand in tall cylinders until relatively clear supernatant fluid can be decanted. Restore to volume with water. Add salt and stir to dissolve. Heat. Add agar and stir vigorously. Heat to boiling to dissolve agar. Check volume and add water if necessary. Dispense in 250-ml amounts in 16-oz screw-capped bottles.

Brain-Heart Infusion Agar

Calf brains, infusion from	200.0 g
Beef heart, infusion from	250.0 g
Proteose *or* Gelysate pancreatic digest of gelatin	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Agar	15.0 g
Distilled or demineralized water	1.0 liter

Final pH 7.4

Dispense and autoclave at 121°C for 15 minutes. For *Actinomyces*, the use of freshly poured plates is recommended.

Brain Heart Infusion Broth

As above, but without agar.

Additives

a. For yeasts: Add Neopeptone, 5 g/liter.

b. For fungi: Add Neopeptone, 5 g, and dextrose, 20 g/liter.

c. For sheep blood medium: Heat 1 liter of broth to boiling. Add 10 percent defibrinated sheep blood while hot; shake for 5 minutes. Filter through Schleicher and Schueel filter paper. Dispense the filtrate in 10-ml quantities in 12.5-cm screw-capped tubes. Autoclave at 120°C for 15 minutes.

Brilliant Green Agar

Proteose No. 3 *or* Polypeptone	10.00 g
pancreatic digest of casein USP	(5.00 g)
peptic digest of animal tissue USP	(5.00 g)

Yeast extract	3.00 g
Sodium chloride	5.00 g
Lactose	10.00 g
Sucrose	10.00 g
Agar	20.00 g
Phenol red (4 ml per liter of 2 percent phenol red prepared by dissolving 1 g in 40 ml 0.1 N NaOH and q.s. to 500 ml with distilled water)	0.08 g
Brilliant green (2 ml per liter of 0.5 percent brilliant green)	12.50 ml
Distilled water	1.00 liter

Final pH 6.9 ± 0.2

Autoclave at 121°C for 15 minutes. Cool and pour 25 to 30 ml per plate. Store in dark.

Brilliant Green Lactose Bile Broth

Peptone	10.0 g
Lactose	10.0 g
Oxgall, dehydrated	20.0 g
Brilliant green (0.1 percent)	13.3 ml
Distilled water, q.s.	1.0 liter

Dissolve the peptone and lactose in about 500 ml distilled water. Dissolve the oxgall in about 200 ml distilled water. The oxgall solution should be pH 7.3 ± 0.2. Combine the above sufficient distilled water to make the volume solutions. Add the brilliant green solution. Add 1 liter. Filter through cotton. Distribute in fermentation tubes and sterilize at 121°C (15 pounds pressure) for 15 minutes. The reaction after sterilization should be not less than pH 7.1 and not more than pH 7.4.

Carlquist Ninhydrin Base

Pancreatic digest of casein USP	15 g
Potassium phosphate	2 g
Dextrose	1 g
Distilled water	1 liter

Dissolve and dispense in 5 ml amounts in screw-capped tubes. Autoclave at 121°C for 15 minutes. Store until use.

Castaneda's Blood Culture Medium (Modified)

The following media are prepared and dispensed as described below:

a. Tryticase soy agar

Dehydrated trypticase soy agar	32 g
Agar, granulated	12 g
Sodium citrate	4 g
Distilled water	800 ml

Suspend the ingredients in the distilled water. Bring to a boil with frequent mixing to dissolve completely.

b. Trypticase soy broth

Dehydrated trypticase soy broth	30 g
Sodium citrate	5 g
Distilled water	1 liter

Add the ingredients to the distilled water and mix well to effect solution.

c. Thioglycollate broth

Dehydrated thioglycollate broth (without indicater)	37.5 g
Sodium citrate	6.3 g
Distilled water	1250.0 ml

Suspend the ingredients in the distilled water and bring to a boil with frequent mixing to dissolve completely.

The melted trypticase soy agar (a) is dispensed in 15-ml amounts to clear, nonchipped, clear-glass, screw-capped bottles of 2-oz capacity.

Rubber diaphragm stoppers are inserted in the screw caps which are subsequently applied loosely over the bottles. The thioglycollate broth (c) is dispensed to a second group of 2-oz bottles in 50-ml amounts and the stoppers and caps similarly applied.

The trypticase soy broth (b) is dispensed in 100-ml amounts for easy handling after sterilization. In addition to the media, a standard dispensing burette (50 ml) is prepared by attaching a 12 to 15 inch length of latex tubing to its bottom end. In the other end of the rubber tubing, a needle holder is inserted and fitted with a 1½ inch, 21-gage needle. The top orifice of the burette is plugged with cotton and covered with foil. The airway of the burette is then covered with gauze and cotton and secured with a rubber band. After inserting the needle holder and needle in a test tube and packing cotton around the rubber tubing, the unit is ready for sterilization.

The dispensing burette and all the media are sterilized at 15 pounds pressure (121°C) for 15 minutes. Immediately after the steam pressure reaches zero, the autoclave is opened and the screw caps of all bottles tightened securely. Asbestos gloves can be used to protect the hands. The agar bottles are inclined on their sides, allowing the melted medium to solidify to a long slant.

After solidification of the slant bottles, the burette is clamped into a ring stand and aseptically filled with the sterile trypticase soy broth. The screw cap of each agar bottle is removed and 15 ml of broth dispensed therein. This is accomplished by plunging the burette needle through the diaphragm stopper and immediately replacing the screw cap. The bottles will possess a sufficient vacuum to allow easy addition of the broth.

The completed agar slant-broth bottles are incubated at 37°C for 48 hours to check for

sterility. It is not necessary to preincubate the thioglycollate culture bottles. All the vacuum culture bottles so prepared may be stored at room temperature.

Casman Broth Base

Proteose peptone No. 3	10.0 g
Tryptose	10.0 g
Beef extract	3.0 g
Nicotinamide	0.05 g
p-Aminobenzoic acid	0.05 g
Dextrose	0.5 g
Corn starch	1.0 g
Sodium chloride	5.0 g
Distilled water	1.0 liter

Mix the ingredients into the cold water and heat to dissolve. Dispense in 10 ml amounts into tubes and sterilize in the autoclave for 15 minutes at 15 pounds pressure (121°C). Cool to 45°C and add 0.5 ml sterile defibrinated rabbit blood to each tube and mix well.

Casman Medium Base

To Casman Broth Base Medium add 14.0 g washed agar (Special Agar, Noble).

Hydrate the ingredients in the 1000 ml of water and heat to boiling to dissolve the medium completely. Sterilize in the autoclave for 15 minutes at 15 pounds of pressure (121°C). Final pH 7.3.

Chocolate Agar Plates and Slants

Agar to which blood has been added and heated until the blood becomes brown or chocolate in color is termed chocolate agar. Chocolate agar is satisfactorily prepared as follows:

Prepare double strength proteose No. 3 agar or G. C. medium base. After sterilization, cool the medium to 48°C in a water bath. Add 5 to 10 percent defibrinated blood and mix well, being careful not to cause bubbles to form. Heat the medium in a boiling water bath, with intermittent gentle swirling of the flask. When the blood is completely browned (usually at 75° to 80°C) remove from the water bath and cool again to 48°C. Aseptically add 2 ml of Bacto supplement A or B (sterile yeast concentrate) per 100 ml of medium. Both supplements are identical except that supplement A contains crystal violet to make the medium inhibitory to Gram-positive organisms.

After mixing, dispense to sterile Petri dishes. Plates may be prepared individually from previously tubed agar or from flasks of 500 or 1000 ml of medium if large numbers are needed. More reliable results will be obtained if plates are freshly prepared. All plates should be preincubated overnight to insure sterility.

Chocolate agar slants may be prepared individually from infusion agar slants by the same method described above, slanting the medium after the blood has turned chocolate brown. To prepare a large number of slants, use a flask containing 50 to 100 ml of agar, proceeding as above. Distribute agar into sterile tubes in 5-ml amounts and slant.

Purpose

Chocolate agar prepared in this manner is an ideal medium for cultivation of *Neisseria gonorrhoeae* from urine, urinary exudates, or other clinical materials. The medium is also successfully used in primary isolation of *Neisseria meningitidis* and *Haemophilus influenzae* from spinal fluid, throat and nasopharyngeal swabs, and other exudates or transudates.

Chopped Meat Medium

Ground meat (fat free)	500 g
Distilled water	1000 ml
1 normal NaOH	25 ml

Use lean beef or horse meat. Remove fat and connective tissue before grinding. Mix meat, water and NaOH and bring to a boil while stirring. Cool, refrigerate overnight, and skim off any remaining fat. Filter the mixture through two layers of gauze, and spread the meat particles out to partially dry.

Add sufficient distilled water to the filtrate to restore 1 liter original volume and add:

Trypticase or peptone	30 g
Yeast extract	5 g
Potassium phosphate	5 g

Dispense meat particles in 16-x 125-mm screwcap tubes with a small scoop and add the enriched filtrate. Use about one part meat particles plus three to four parts liquid (v/v) per tube. Add a few iron filings to each tube. Tubes should be over half full (about 8 ml fluid). Autoclave at 121°C for 15 minutes.

Chopped Meat-Dextrose

Add 3 g glucose per liter chopped meat filtrate before dispensing.

Chopped Meat-Dextrose-Starch

Add 2 g soluble starch per 1000 ml chopped meat-dextrose filtrate before dispensing.

Chopped Meat-Starch

Add 2 g soluble starch per 1000 ml chopped meat filtrate before dispensing.

Chopped Meat Agar Slants For Spores

Add 15 g agar per 1000 ml chopped meat

medium. Boil to melt agar, dispense in tubes, autoclave and slant.

Citrate Agar, Christensen

Christensen citrate sulfide agar

Sodium citrate	3.0	g
Dextrose	0.2	g
Yeast extract	0.5	g
Cysteine hydrochloride	0.1	g
Ferric ammonium citrate	0.4	g
Monopotassium phosphate	1.0	g
Sodium chloride	5.0	g
Sodium thiosulfate	0.08	g
Phenol red	0.012	g
Agar	15.0	g
Distilled water	1.0	liter

Final pH 6.7

Coagulase Plasma (DIFCO) (Bacto-Coagulase Plasma)

Commercial source in lieu of fresh human citrated plasma.

Cooked Meat Medium With Petrolatum Seal

Cut 500 g of lean beef or veal heart into small cubes and cover with distilled water. Bring to a boil and simmer over a low flame for 1 hour. Strain off and set aside the fluid infusion. Pass the meat three times through a meat grinder and then break up the particles by rubbing them between the hands.

To the fluid infusion add sufficient distilled water to make 2 liters and mix it with the meat. Add one normal sodium hydroxide to the solution until the reaction of the supernate is pH 8.0. Weigh the medium in a tared vessel and then autoclave it for 15 minutes at 121°C at 15 pounds pressure. Restore the weight with distilled water. Readjust the reaction to pH 8.0. Boil for 10 minutes. Restore the weight with water and readjust the reaction to pH 8.0. Boil again for 10 minutes. Restore the weight and if the reaction is more acid than pH 7.5, readjust to this reaction.

Distribute the medium into tubes, keeping the mixture well stirred so that there may be a uniform deposit of meat particles in each tube. Cover with a layer of sterile petrolatum about 7 mm thick and sterilize in the autoclave. Check the final reaction, which should be about pH 7.1. (The reaction must not be acid at this point.)

The repeated adjustment of reaction and boiling is necessary because the supernatant fluid contains little buffer, whereas the meat particles slowly take up large amounts of alkali. The reaction of the mixture is difficult to stabilize.

Not all petrolatum jellies are of the right consistency or melting point for the purpose of sealing anaerobic media. The melting point should be above 45°C. If bacterial spores are present in petrolatum, they are not subjected to steam (moist heat) and the temperature of the autoclave at 18 pounds pressure is only about 125°C. Before use, therefore, the petrolatum should be sterilized in the oven (dry heat) at 165°C for 2 hours. If this procedure is followed, contamination can be avoided.

Cystine Heart Agar Plus Blood and Antibiotics

Beef heart, infusion from	500	g
Proteose or Polytone	10	g
Dextrose	10	g
Sodium chloride	5	g
L-Cystine	1	g
Agar	15	g
Distilled Water	1	liter

Final pH 6.8

Mix and heat to obtain complete solution. Autoclave at 121°C for 15 minutes. Cool to 45° to 48°C. Aseptically add 25 ml of packed human blood cells or add 50 ml of defibrinated rabbit blood. Aseptically add antibiotics—1 ml each of solutions containing: pencillin, 100,000 units/ml; polymyxin B sulfate, 100,000 units/ml; and cycloheximide, 0.1 mg/ml.

Cystine Tryptophan Agar (CTA) Medium

Cystine	0.5	g
Pancreatic digest of casein USP	20.0	g
Agar	2.5	g
Sodium chloride	5.0	g
Sodium sulfite	0.5	g
Phenol red	0.017	g
Distilled water	1.0	liter

Final pH 7.3

Mix and heat with agitation until solution occurs. Dispense and autoclave at 115° to 118°C for 15 minutes.

Cycloheximide Chloramphenicol Agar

Myocosel agar, Mycobiotic agar, etc.

Sabouraud dextrose agar	1.00	liter
Ajar	5.00	g
Cycloheximide, in 10 ml of acetone	0.50	g
Chloramphenicol, in 10 ml of 95 percent alcohol	0.05	g

Heat to dissolve the agar and the Sabouraud dextrose agar. Dispense in tubes and autoclave at 121°C for 10 minutes. Cool in a slanted position.

Deoxyribonuclease (DNase) Test Agar

Deoxyribonucleic acid	2	g

Pancreatic digest of casein USP	15 g
Papaic digest of soy meal USP	5 g
Sodium chloride	5 g
Agar	15 g
Distilled water	1 liter

Final pH 7.3

Esculin Agar, Modified

Esculin	1.0 g
Ferric citrate	0.5 g
Heart infusion agar (blood agar base)	40.0 g
Distilled water	1.0 liter

Heat to dissolve. Cool to 55°C; adjust pH to 7.0. Dispense in 5-ml amounts in 16 by 125 mm, cotton-plugged tubes. Autoclave at 121°C for 15 minutes. Cool in a slanted position.

Esculin Broth

Peptone or Gelysate	5.0 g
Dipotassium phosphate	1.0 g
Andrade indicator (Andrade carbohydrate broths)	10 ml

Fermentation Base*

Thioglycollate medium without dextrose or indicator. For each 1000 ml medium, add:

Yeast extract (Difco)	2 g
Bromthymol blue (1 percent aqueous solution)	1 ml

Dispense 8 ml in 15-x 125-mm tubes with fermentation vials.

*Available commercially as Bacto CHO base (Difco C841-01).

Flagella Broth

Bacto tryptose	10 g
NaCl C.P.	2.5 g
K_2HPO_4	1.0 g
Distilled water	1.0 liter

Adjust pH to 7.0. Dispense in 15-x 125-mm tubes, 8.0 ml per tube. Autoclave at 121°C for 15 minutes.

Fildes Enrichment Agar

Sodium chloride, 0.85 percent	150 ml
Hydrochloric acid	6 ml
Defibrinated sheep blood	50 ml
Pepsin, granular	1 g

Mix in a glass-stoppered bottle. Shake thoroughly. Place in a water bath at 55°C for 4 hours; shake occasionally. Add about 6 ml of 5 N sodium hydroxide to obtain a pH of 7.0 or slightly less. Add 0.25 ml chloroform and shake thoroughly. Store at 4°C. Before use, gently heat in water bath to remove chloroform. Add 4 to 10 ml of enrichment to 200 ml of sterile nutrient agar melted and cooled to 56°C. Pour into plates.

Fletcher Semisolid Medium

Pancreatic digest of casein USP or pancreatic digest of gelatin	0.3 g
Beef extract	0.2 g
Sodium chloride	0.5 g
Agar	1.5 g
Water	920.0 ml

Final pH 7.4 to 8.0

Suspend ingredients in cold water and heat to boiling to dissolve. Autoclave at 121°C for 15 minutes. Cool to 56°C and add filter-sterilized rabbit serum. Dispense in screw-capped tubes in 5- to 7-ml amounts. Inactivate the medium by placing the tubes in a water bath at 56°C for 1 hour on two successive days.

Formate Ricinoleate Broth

Peptone	5 g
Lactose	5 g
Sodium Formate	5 g
Sodium Ricinoleate	1 g
Distilled water	1 liter

Place the above materials in 1 liter of distilled water. Heat slowly in a water bath with constant stirring, until dissolved. Adjust the reaction so that the pH reading after sterilization will be 7.3 to 7.5. Distribute in fermentation tubes and autoclave at 11 to 13 pounds pressure for 15 minutes.

Gelatin Medium

Extract or infusion broth	425 ml
Gelatin	50 g

Mix gelatin and broth thoroughly and heat in a boiling water bath until the gelatin is dissolved. Adjust the reaction to pH 7.6 to 7.8. Tube and autoclave at 15 pounds pressure (121°C) for 15 minutes.

Gelatin Medium, For Anaerobes

Thioglycollate medium without indicator plus hemin	1 liter
Gelatin	50 g

Mix and heat with agitation to dissolve. Dispense in screw-capped tubes filled at least half full and autoclave at 118°C for 15 minutes.

NOTE. Alternatively, use dehydrated thioglycollate gelatin (Thiogel) medium plus hemin.

Gluconate Broth (Shaw and Clark)

Peptone	1.5 g
Yeast extract	1.0 g
Potassium phosphate, dibasic	1.0 g
Potassium gluconate	40.0 g
Distilled water	1.0 liter

Dissolve the solids in the water with heat and dispense. Sterilize at 121°C for 15 minutes.

Sodium gluconate (37.25 g) can be used instead of the potassium gluconate given above.

Glucose and Other Fermentation Sugars

Add 6 g glucose to 1000 ml fermentation base before dispensing and autoclaving. Use 8.5 ml in 15-x 125-mm tubes with fermentation vials.

Arabinose, Glycerol, Lactose, Maltose, Mannitol, Rhamnose, Sucrose, Trehalosh, Xylose

To 8.0 ml autoclaved fermentation base in 15-x 125-mm tubes, add 0.5 ml Seitz filtered aqueous solution of 10 percent carbohydrate. Use sterile technique.

Salicin

To 8.0 ml autoclaved fermentation base in 15-x 125-mm tubes, add 1.0 ml Seitz filtered aqueous solution of 5 percent salicin. Use sterile technique.

Gohar Medium

Peptone	10 g
Potassium tellurite, 1 percent	2 ml
Distilled water	1 liter

Final pH 8.0

Dissolve the peptone in the distilled water and adjust to a pH of 8.3. Autoclave at 15 pounds pressure (121°C) for 15 minutes and aseptically add the filter-sterilized potassium tellurite solution. Distribute in 3 to 5-ml amounts to sterile, cotton-plugged test tubes.

Purpose

Gohar broth is an alkaline enrichment broth for primary isolation of Vibrio cholerae from "rice-water stools." Potassium tellurite is included to inhibit growth of enteric Gram-negative bacilli other than V. cholerae.

GN Broth

Gram-Negative Enrichment Broth	
Pancreatic digest of casein	10.0 g
Peptic digest of animal tissue	10.0 g
Dextrose	1.0 g
D-Mannitol	2.0 g
Sodium citrate	5.0 g
Sodium desoxycholate	0.5 g
Dipotassium phosphate	4.0 g
Monopotassium phosphate	1.5 g
Sodium chloride	5.0 g
Distilled water	1.0 liter

Final pH 7.0

Dissolve, dispense, and sterilize by autoclaving at 116°C for 15 minutes. Alternatively, steam for 30 minutes at 100°C.

Hektoen Enteric Agar

Pepton, e.g., Peptic digest of animal tissue USP	12.0	g
Yeast extract	3.0	g
Bile salts	9.0	g
Lactose	12.0	g
Sucrose	12.0	g
Salicin	2.0	g
Sodium chloride	5.0	g
Sodium thiosulfate	5.0	g
Ferric ammonium sulfate	1.5	g
Bromothymol blue	0.064	g
Acid fuchsin	0.10	g
Agar (dried)	13.5	g
Water	1.0	liter

Heat with agitation until solution occurs; do not autoclave. Cool to 45° to 50°C and pour into plates.

Hydrogen Peroxide, 30 Percent

Hydrogen peroxide, 30 percent	10 ml
Distilled water	90 ml

Mix distilled water with peroxide in an Erlenmyer flask by inverting several times. Store in brown bottle in the refrigerator.

Hydrogen Sulfide (H_2S)

Tryticase (BBL)	1.00 g
Yeast extract	0.50 g
Agar	0.20 g
Lead acetate, 10 percent solution	0.20 ml
Glucose	0.50 g
Distilled water	100.00 ml

Adjust pH to 7.2 to 7.3. Dispense 8 ml in 15-x 125-mm tubes. Autoclave at 121°C for 15 minutes.

Indol-Nitrite (BBL)

Prepare as directed by manufacturer. Dispense 8.0 ml in 15-x 125-mm tubes.

Infusion Agar Slants

Use blood agar base or another comparable medium. Dispense in 15-x 125-mm tubes. Autoclave and slant.

Infusion Agar-Glucose Slants

Add 1 g glucose per 100 ml infusion agar. Autoclave and slant.

Inulin Fermentation Disk (DIFCO)

Available commercially. (Not prepared in laboratory.)

Iron Milk

Place a few iron filings in the bottom of 15-x 125-mm test tubes. Add 8 ml whole, nonhomo-

genized milk to each tube. Autoclave at 121°C for 15 minutes.

Kliger Iron Agar

Peptone	20.0	g
Meat extract (optional)	3.0	g
Yeast extract (optional)	3.0	g
Lactose	10.0	g
Dextrose	1.0	g
Sodium chloride	5.0	g
Ferric ammonium citrate	0.5	g
Sodium thiosulfate	0.5	g
Agar	12 to 15.0	g
Phenol red	0.025	g
Water	1.0	liter

Final pH 7.4

Mix and heat with agitation until solution occurs. Dispense for deep slants and autoclave at 121°C for 15 minutes.

Koser Citrate Medium (Broth)

Sodium chloride	5.0	g
Dipotassium phosphate	1.0	g
Ammonium dihydrogen phosphate	1.0	g
Magnesium sulfate	0.2	g
Sodium citrate	2.0	g
Distilled water	1.0	liter

Dissolve salts in water and filter to a clear solution. Dispense 5 ml per tube and sterilized at 121°C for 15 minutes. This medium is available commercially.

Lactose-Motility Medium

Rehydrate LM agar (Difco) as directed. Dispense 8 ml in 15-x 125-mm tubes. Autoclave at 121°C for 10 minutes.

Levinthal's Agar and Broth

Brain heart infusion broth	100	ml
Heat to boiling, and add:		
Defibrinated horse blood	10	ml
Peptone phosphate agar **or** Proteose No. 3 agar	45	g
Agar	15	g
Distilled water	1	liter

Dispense the agar base in 100-ml amounts and autoclave. Cool. Mix equal parts of sterile, fluid agar base and the horse blood broth. Pour into plates.

Listeria Medium

Peptone: Tryptose *or* Biosate	10.0	g
Beef extract	3.0	g
Sodium chloride	5.0	g
Glycine anhydride	10.0	g
Lithium chloride	0.5	g
Phenylethanol	2.5	g
Agar	15.0	g
Distilled water	1.0	liter

Final pH without blood, 7.3

Autoclave at 121°C for 15 minutes. Cool to 45° to 50°C and add sterile defibrinated sheep blood to a final concentration of 5 percent.

Loeffler's Medium

Beef extract	3.0	g
Peptone	10.0	g
Sodium chloride	5.0	g
Dextrose	10.0	g
Demineralized water	1.0	liter

Combine and dissolve ingredients by heating. Cool to 55°C. Add 3 liters of filtered horse serum through a Buchner funnel containing infusorial earth filter. Sterilize by filtration and dispense aseptically, 2 to 3 ml in 16 × 100 mm screw-capped tubes. Do not flame tubes, and avoid formation of bubbles. Slant and inspissate at 95° to 98°C for 50 minutes. Cool and then tighten the caps. Incubate for 24 hours at 35° to 37°C and for 48 hours at 20°C. Store at 4° to 6°C.

Lowenstein-Jensen Medium

Monopotassium phosphate, anhydrous	2.4	g
Magnesium sulfate : $7H_2O$	0.24	g
Magnesium citrate	0.6	g
Asparagine	3.6	g
Potato flour	30.0	g
Glycerol	12.0	ml
Distilled water	600.0	ml
Homogenized whole eggs	1.0	liter
Malachite green, 2 percent aqueous	200.0	ml

Dissolve the salts and asparagine in the water. Admix the glycerol and potato flour, autoclave at 121°C for 30 minutes, and cool to room temperature. Scrub eggs, not more than 1 week old, in 5 percent soap solution. Allow to stand for 30 minutes in soap solution; then rinse thoroughly in cold running water. Immerse in 70 percent ethyl alcohol for 15 minutes. Break eggs into a sterile flask. Homogenize by hand shaking and filter through four layers of gauze. Add 1 liter of homogenized eggs to the potato-salt mixture. Prepare the malachite green and admix thoroughly. Dispense 6 to 8 ml in 20 by 150 screw-capped tubes. Slant and inspissate at 85°C for 50 minutes. Incubate for 48 hours at 37°C to check sterility and store at 4° to 6°C with caps tightly closed.

Lysine Iron Agar

Bacto peptone *or* Gelysate pancreatic digest of gelatin	5.00	g
Yeast extract	3.00	g
Dextrose	1.00	g

L-Lysine	10.0	g
Ferric ammonium citrate	0.5	g
Sodium thiosulfate	0.04	g
Bromocresol purple	0.02	g
Agar	15.0	g
Distilled water	1.0	liter

Final pH 6.7

Dispense in 4-ml amounts and autoclave at 121°C for 12 minutes. Cool in slanted position to form slants with deep butts. Inoculate with a straight wire by stabbing to the base of the butt and by streaking the slant. Incubate at 37°C for 18 to 24 hours. The medium was designed originally for the examination of blackened colonies on bismuth sulfite agar plates.

MacConkey Agar

Bacto peptone *or* Gelysate	17.0	g
Peptone: Proteose *or* Polypeptone	3.0	g
Lactose	10.0	g
Bile salts	1.5	g
Sodium chloride	5.0	g
Agar	13.5	g
Neutral red	0.03	g
Crystal violet	0.001	g
Distilled water	1.0	liter

Final pH 7.1

Sucrose may be added to give a final concentration of 1 percent. Autoclave at 121°C for 15 minutes. Use as plates or as slants.

Malonate Broth, Modified

Yeast extract	1.0	g
Ammonium sulfate	2.0	g
Dipotassium phosphate	0.6	g
Monopotassium phosphate	0.4	g
Sodium chloride	2.0	g
Sodium malonate	3.0	g
Dextrose	0.25	g
Bromothymol blue	0.025	g
Distilled water	1.0	liter

Final pH 6.7

Autoclave at 121°C for 15 minutes. Inoculate from a young agar slant or preferably with a 3-mm loopful of a broth culture. Incubate at 37°C and observe after 24 and 48 hours. Positive results are indicated by a change in the color of the indicator from green to Prussian blue.

Mannitol Salt Agar, USP

Beef extract	1.0	g
Peptone *or* Polypeptone	10.0	g
Sodium chloride	75.0	g
Mannitol	10.0	g
Agar	15.0	g
Phenol red	0.025	g
Distilled water	1.0	liter

Final pH 7.4

Heat to dissolve and autoclave at 121°C for 15 minutes.

Meat Extract Agar

Nutrient Agar Modified

For Vibrio cholerae

Beef extract	3	g
Peptone *or* Gelysate pancreatic digest of gelatin	10	g
Sodium chloride	5	g
Agar (not dried)	20	g
Distilled water	1	liter

Adjust pH to 7.6 with 1 N sodium hydroxide. Autoclave at 121°C for 15 minutes. Cool to 45° to 50°C and dispense in Petri dishes.

Meat Extract Broth

Meat extract	3	g
Sodium chloride	5	g
Peptone	10	g
Distilled water	1	liter

Mix ingredients and heat to a temperature below 60°C until dissolved. Heat the mixture in boiling water bath for 20 minutes. Adjust the reaction to pH 7.6 and heat again for 15 minutes as before. Filter the broth through paper. Distribute into test tubes or flasks and autoclave at 15 pounds pressure (121°C) for 15 minutes.

Meat Infusion Media

Ground lean beef, veal or pork	500.0	g
Peptone	10.0	g
Sodium chloride	5.0	g
Distilled water	1.0	liter

Mix meat and water and let stand for 1 hour at room temperature. Boil for 5 minutes, strain through gauze, and filter through paper. Add peptone and salt, stir until the peptone is dissolved, and adjust to pH 7.8 to 8.0. Boil for 20 minutes and q.s. to one liter. Filter through paper. Distribute into tubes or bottles and sterilize. (Agar (15.0 g) for solid medium.)

Methylene Blue Milk

Skimmed or centrifuged whole milk	500	ml
Methylene blue, aqueous solution, 1 percent (sterile)	10	ml

Autoclave the milk at 10 pounds pressure (115°C) for 15 minutes. Aseptically add the sterile aqueous solution of methylene blue. Tube the medium aseptically in 5-ml amounts in suitably sized test tubes.

Purpose

The reduction of methylene blue in this medium is used for the identification of certain species of enterococci.

Middlebrook and Cohn 7H10 Agar (with Oleic Acid, Dextrose, Citrate)

Stock Solutions

Solution 1. Store at room temperature.

Monopotassium phosphate	15.0 g
Disodium phosphate	15.0 g
Distilled water	250.0 ml

Solution 2. Store at 4° to 10°C.

Ammonium sulfate	5.0 g
Monosodium glutamate	5.0 g
Sodium citrate · $2H_2O$ USP	4.0 g
Ferric ammonium citrate	0.4 g
Magnesium sulfate ($7H_2O$) ACS	0.5 g
Biotin, in 2 ml of 10 percent NH_4OH	5.0 mg
Distilled water to	250.0 ml

Solution 3. Store at 4° to 10°C.

Calcium chloride · $2H_2O$ ACS	50.0 mg
Zinc sulfate · $7H_2O$ ACS	100.0 mg
Copper sulfate · $5H_2O$ ACS	100.0 mg
Pyridoxine hydrochloride	100.0 mg
Distilled water to	100.0 ml

Solution 4. Glycerol, reagent.

Solution 5. Malachite green, 0.01% aqueous.

To 975 ml of distilled water, add:

Solution 1	25.0 ml
Solution 2	25.0 ml
Solution 3	1.0 ml
Solution 4	5.0 ml

Adjust pH to 6.6 by adding approximately 0.5 ml of 6 N HCl. Add 2.5 ml of solution 5 and 15 g of agar. Autoclave at 121°C for 15 minutes. Cool to 56°C and add 100 ml of O A D C Enrichment:

Bovine albumin fraction V	50.0 g
Sterile saline, 0.85 percent	900.0 ml

Dissolve and add 30 ml of the following:

Oleic acid	0.6 ml
Distilled water to	30.0 ml
Sodium hydroxide, 6 M	0.6 ml

Warm to 56°C: swirl until solution occurs.

Adjust reaction to pH 7.0, measured electrometrically. Add 40 ml of sterile 50 percent aqueous dextrose. Sterilize by filtration. Heat at 56°C for 1 hour, incubate overnight, and heat again at 56°C; again incubate overnight at 37°C for sterility. Add 100 ml of this solution to the agar base. Add also 2 ml of freshly prepared, membrane filter-sterilized, catalase solution containing 1,000 µg/ml. Dispense the complete medium into Petri dishes or tubes. Store at 4° to 8°C; unsealed and unprotected containers should be used for no longer than 1 week. During preparation and storage, protect from light.

Motility Medium

Use Motility Test Medium (BBL) or Motility Medium (Difco) diluted to a final concentration of 0.4 percent agar as follows:

Motility medium (Difco)	16.0 g
Nutrient broth	4.0 g
NaCl	1.0 g
Distilled water	1000.0 ml

Dispense 8 ml in 15-x 125-mm tubes. Autoclave at 121°C for 15 minutes.

Motility Test Medium

Beef extract	3 g
Peptone	10 g
Sodium chloride	5 g
Agar	4 g
Distilled water	1 liter

Dissolve the ingredients in the distilled water and adjust the pH to 7.4. Tube using approximately 8 ml per tube, and sterilize at 121°C for 15 minutes.

MR-VP Medium

Dipotassium phosphate	5.0 g
Polypeptone	7.0 g
Glucose	5.0 g
Distilled water	1.0 liter

Suspend ingredients in water and heat slightly to dissolve. Place 10 ml in tubes and sterilize at 121°C for 10 minutes at 15 pounds pressure. The medium should be water clear; if yellow, it is unsatisfactory for use.

Muellar-Hinton Agar

Beef, infusion from	300.0 g
Acid hydrolysate of casein	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled water	1.0 liter

Final pH 7.4

Dispense and autoclave at 116° to 121°C for 15 minutes. Cool to 45° to 50°C and add blood, if desired.

Mueller-Hinton Broth

As above, but with omission of agar.

Nutrient Agar

Beef extract	3.0 g
Peptone or Gelysate pancreatic digest of gelatin	5.0 g
Agar	15.0 g
Distilled water	1.0 liter

Mix ingredients and autoclave at 121°C for 15 minutes.

Ornithine Decarboxylase

Peptone	5.0 g
Yeast extract	3.0 g
Dextrose	1.0 g
L-Ornithine	5.0 g
Brom Cresol Purple	0.02 g

Rehydrate the ingredients in 100 ml of cold, distilled water and warm to dissolve completely. It is best to add the L-ornithine last. Dilute to 1000 ml. Ornithine HC1 being highly acidic, requires adjustment of pH with sodium hydroxide before sterilization.

Usually one liter of medium in which 5 grams of Ornithine HC1 is dissolved requires the addition of 2.1 ml of 10 N NaOH. Dispense in 5 ml amounts into screw-capped culture tubes and sterilize in autoclave 15 minutes at 15 pounds pressure (121°C). Medium is purple, pH 6.5

Oxidation-Fermentation Test Medium of Basal Medium

Peptone, that is, pancreatic digest of casein	2.0 g
Sodium chloride	5.0 g
Dipotassium phosphate	0.3 g
Bromothymol blue	0.03 g
Agar	3.0 g
Distilled water	1.0 liter

Final pH 7.1

Dispense 3 or 4 ml per 13 by 100 mm test tube. Autoclave at 121°C for 15 minutes. Cool and add 10 percent dextrose solution in distilled water for a final concentration of 1 percent dextrose. Other carbohydrates may be substituted for dextrose, if desired. Dispense aseptically.

Peptone Water (Dunham Solution)

Peptone	10 g
Sodium chloride (NaCl)	5 g
Distilled water	1000 ml

Mix ingredients and heat slowly until the peptone is dissolved. Adjust to a pH of 8.0. Tube and sterilize in the autoclave.

Purpose

Peptone water is a medium used for cultivation of Vibrio cholerae from stools.

Peptone-Yeast Extract (PY) Medium

Peptone	20.0 gm
Yeast extract (Difco)	10.0 gm
Salts solution*	40.0 ml
Cysteine HCl	0.5 gm
Resazurin solution**	4.0 ml
Distilled water	1000.0 ml

Adjust pH to 7.2, dispense 8 ml in 15-x 125-mm screwcap tubes, and autoclave at 121°C for 15 minutes.

*Salts solution:

$CaCl_2$ (anhydrous)	0.2 gm
$MgSO_4$	0.2 gm
K_2HPO_4	1.0 gm
KH_2PO_4	1.0 gm
$NaHCO_3$	10.0 gm
NaCl	2.0 gm

Mix $CaCl_2$ and $MgSO_4$ in 300 ml distilled water until dissolved. Add 500 ml water, and while swirling slowly add remaining salts. Continue swirling the salts until all are dissolved. Add 200 ml distilled water, mix, and store at 4°C.

**Resazurin solution:

Dissolve one resazurin tablet (ca. 11 mg Allied Chemical catalog #506) in 44 ml distilled water.

Peptone-Yeast Extract-Glucose (PYG) Medium

Add 1.0 gm glucose to 100 ml of PY medium. Adjust to pH 7.2, dispense, and autoclave at 121°C for 15 minutes.

Phenol Red Broth

Peptone, carbohydrate-free	5.0 g
Sodium chloride	5.0 g
Phenol red	0.018 g
Distilled water	1.0 liter

Final pH 7.4

Mix solids in water and heat to boiling to dissolve the components completely. Sterilized at 115°C to 118°C at 12 pounds for 10 minutes. Add filtered, sterilized carbohydrate solution as desired (0.1 percent). Dispense in 5-ml amounts to each tube and cap or plug with cotton. Store at room temperature.

Phenylalanine Agar

DL-phenylalanine	2 g
Yeast extract	3 g
Disodium phosphate	1 g
Sodium chloride	5 g
Agar	20 g
Distilled water	1 liter

Dissolve the solids in water with heat. Dispense in tubes and sterilize at 121°C for 10 minutes. Let solidify in a position that produces a long slant. The medium is available commercially.

Potassium Cyanide Medium (KCN)

Peptic digest of animal tissue USP	10.0 g
Sodium chloride	5.0 g
Monopotassium phosphate	0.225 g
Disodium phosphate	5.64 g

Distilled water	1.0	liter

Adjust to final pH of 7.6 and autoclave at 121°C for 15 minutes. Refrigerate until thoroughly chilled. To the cold medium add 15 ml of a 0.5 percent KCN solution prepared in cold sterile distilled water. Dispense about 1 ml per small sterile tube. Stopper quickly with corks sterilized in heated paraffin. (May be stored at 4°C for weeks.) Inoculate with one loopful of a 24-hour broth culture grown at 37°C. Incubate at 37°C and observe daily for 2 days. Positive results are indicated by growth in the presence of KCN.

Schuffner's Medium

Peptone (Proteose No. 3)	1	g
Ringer solution	200	ml
Sorensen phosphate buffer, pH 7.2	100	ml

Dissolve in 1 liter distilled water. Boil until the phosphates have been precipitated and filter after cooling. Distribute into small tubes. Sterilize at 100°C. The pH is 6.9 to 7.0. Add 10 percent sterile serum and inactivate at 56°C for 30 minutes. Some rabbit sera agglutinate leptospirae and cannot be used in medium; each rabbit serum must be previously tested.

SF Broth

Tryptone	20.0	g
Dextrose	5.0	g
Dipotassium phosphate	4.0	g
Monopotassium phosphate	1.5	g
Sodium azide	0.5	g
Sodium chloride	5.0	g
Brom-Cresol-Purple	0.032	g
Distilled water	1.0	liter

Final pH 6.9

Dissolve the ingredients in the distilled water. Distribute medium in tubes and sterilize in the autoclave at 15 pounds for 15 minutes (121°C).

Simmon's Citrate

Magnesium sulfate	0.2	g
Monoammonium phosphate	1.0	g
Dipotassium phosphate	1.0	g
Sodium citrate	2.0	g
Sodium chloride	5.0	g
Agar	15.0	g
Brom-Thymol-Blue	0.08	g

Final pH 6.8

Mix ingredients in the water and heat to boiling to dissolve the medium completely. Distribute in tubes or flasks and sterilize by autoclaving for 15 minutes at 15 pounds pressure (121°C). Allow the tubed medium to cool in slanting position.

SIMs Medium

Beef extract	3.0	g
Peptone	30.0	g
Peptonized iron	0.2	g
Sodium thiosulfate	0.025	g
Agar	3.0	g
Distilled water	1.0	liter

Final pH 7.3

To rehydrate the medium, suspend the ingredients in the water and heat to boiling to dissolve the medium completely. Distribute the medium in tubes to a depth of about 3 inches and sterilize in the autoclave for 15 minutes at 15 pounds pressure (121°C). The tubes of medium are solidified in a vertical position.

Sodium Hippurate Broth

Trypticase soy broth	500	ml
Sodium hippurate	5	g

Prepare 500 ml of trypticase soy broth and include exactly 5 g (1 percent) of sodium hippurate in the preparation. Dispense in 5-ml amounts to standard culture tubes and with a wax pencil, mark the fluid level of medium on each tube. Autoclave the medium at 15 pounds pressure (121°C) for 15 minutes.

Inoculate the *Streptococcus* sp to be tested to the medium and incubate at 37°C for 48 hours. At the end of the incubation period, restore the fluid loss to the culture by adding distilled water to the wax pencil mark on the tube. Perform the ferric chloride test for detecting hydrolysis of sodium hippurate as described in chapter 14.

Purpose

Hippurate broth is used to differentiate Group B streptococci from Groups A, C, and D. Only Group B strains of these groups will hydrolyze sodium hippurate in culture, which is detected by performing the hippurate test on 48-hour cultures.

SP Hajna's Transport Medium

Yeast extract	1.0	g
Diammonium phosphate	4.0	g
Monopotassium phosphate	2.0	g
Sodium chloride	5.0	g
Magnesium sulfate	0.4	g
Sodium citrate	5.0	g
Sodium desoxycholate	0.5	g
Glycerol	300.0	ml

Final pH 7.0

Dissolve ingredients in 700 ml of distilled water. Add the glycerol and mix. Distribute in tubes or screw-capped jars and sterilize in the autoclave at 10 pounds of pressure (116°C) or at 15 pounds (121°C) for 15 minutes.

Stuart's Transport Medium

Sodium thioglycolate	0.9	g
Sodium glycerophosphate	10.0	g
Calcium chloride USP	0.1	g
Methylene blue	0.002	g
Agar	3.0	g
Distilled water	1.0	liter

Mix the ingredients in cold water (portion of liter) until dissolved. Pour in remaining water and heat to boiling to dissolve to completion. Dispense the hot medium into suitable transport tubes, cap tightly and sterilize for 15 minutes at 15 pounds pressure (121°C).

Tarshis Blood Medium

Agar	1.5 g
Glycerol	1.0 ml
Human bank blood	30.0 ml
Distilled water	69.0 ml
Penicillin (crystalline potassium G)	100 units per ml

Final pH 6.8

Add the agar and glycerol to the water. Heat the mixture to a boil to melt the agar. Autoclave at 15 pounds pressure (121°C) for 15 minutes. Cool the base to 45°C in a water bath and warm the blood in the same bath. Using aseptic technique, add the blood and penicillin to the sterilized base. Gently mix and dispense to sterile screw-capped tubes. Allow the medium to cool in a slanting position.

Purpose

This medium is used for cultivation of Mycobacterium tuberculosis.

Tetrathionate Broth

Prepare tetrathionate broth base as follows:

Proteose peptone	5	g
Bile salts	1	g
Calcium carbonate	10	g
Sodium thiosulfate	30	g
Distilled water	1	liter

Suspend 46 g of the dehydrated base in 1 liter of distilled water. Also add the ingredents listed below:

Brilliant green (1:1000)	10	ml
Bile salts No. 3	4	g
Calcium carbonate	30	g
Sodium thiosulfate	40	g
L-cystine	1	g

Mix the medium thoroughly and heat in a boiling water bath. The calcium carbonate will not dissolve since the concentration is above supersaturation. While keeping the medium well-mixed in a uniform suspension, dispense in 10 ml amounts to standard culture tubes. Immediately before inoculating the broth with specimens, add 6 drops of the following iodine-iodide solution to each tube.

Iodide crystals	6	g
Potassium iodide	5	g
Distilled water	20	ml

Do not autoclave tetrathionate broth. Furthermore, do not heat the medium after adding the iodine-iodide solution.

Purpose

The medium is recommended for primary isolation of *Salmonella* spp from outbreaks of gastroenteritis. The medium is inhibitory to *S. typhi* and *Shigella* spp. When the latter organisms are suspected, a less inhibitory medium such as selenite-F should be employed. The medium is inhibitory to coliform bacilli which allows small numbers of salmonellae to be isolated from fecal materials. After 18 to 24 hours' incubation of tetrathionate broth heavily inoculated (1 g of specimen) with fecal material, the growth is subcultured to a differential plating medium to obtain isolated colonies.

Thayer-Martin Medium (Transport)

Combine sterile solutions with the agar base cooled to 50°C.

GC agar base sterile, double-strength, 100 ml (see Chocolate agars).

Hemoglobin, 2 percent aqueous, 100 ml, *or* Chocolated defibrinated blood, 5 percent.

Antibiotic inhibitors to give final concentrations per 100 ml of medium of: vancomycin, 300 μg; colistin, 750 μg; and nystatin, 1,250 units.

Chemical enrichment, for example, 1 percent IsoVitalix

Vitamin B_{12}	0.01	g
L-Glutamine	10.0	g
Adenine	1.0	g
Guanine hydrochloride	0.03	g
p-Aminobenzoic acid	0.013	g
L-cystine	1.1	g
Dextrose	100.0	g
Diphosphophyridine nucleotide oxidized (coenzyme 1)	0.25	g
Cocarboxylase	0.1	g
Ferric nitrate	0.02	g
Thiamine hydrochloride	0.003	g
Cysteine hydrochloride	25.9	g
Distilled water	1.0	liter

Other supplements, for example, Supplement "B," may be used instead of the defined chemical enrichment.

Thioglycolate Fluid

Pancreatic digest of casein USP	17.0	g
Papaic digest of soy meal USP	3.0	g

Dextrose	6.0	g
Sodium chloride	2.5	g
Sodium thioglycolate	0.5	g
Agar	0.7	g
L-Cystine	0.25	g
Sodium sulfite	0.1	g
Distilled water	1.0	liter

Final pH 7.0

Heat with agitation until the medium boils. Dispense in tubes or container filled half full. Autoclave at 118° to 121°C for 15 minutes.

TCBS Agar

Thiosulfate Citrate Bile Salts Sucrose Agar

Sodium thiosulfate	10.0	g
Sodium citrate	10.0	g
Oxgall	5.0	g
Sodium cholate	3.0	g
Sucrose	20.0	g
Pancreatic digest of casein USP	5.0	g
Peptic digest of casein USP	5.0	g
Yeast extract	5.0	g
Sodium chloride	10.0	g
Iron citrate	1.0	g
Thymol blue	0.04	g
Bromothymol blue	0.04	g
Agar (dried)	14.0	g
Distilled water	1.0	liter

Final pH 8.6

Heat with agitation and boil for 1 minute. Cool to 45° to 50°C and pour into plates. Do not autoclave.

Todd-Hewitt Medium

Beef heart, infusion from	500.0	g
Peptone component	20.0	g
Dextrose	2.0	g
Sodium chloride	2.0	g
Disodium phosphate	0.4	g
Distilled water	1.0	liter

Final pH 7.8

Dissolve, dispense, and autoclave at 121°C for 15 minutes.

Triple Sugar Iron (TSI) Agar

Pancreatic digest of casein USP	10.0	g
Peptic digest of animal tissue USP	10.0	g
or		
Beef extract	(3.0	g)
Yeast extract	(3.0	g)
Peptone components	(20.0	g)
Dextrose	1.0	g
Lactose	10.0	g
Sucrose	10.0	g
Ferrous sulfate *or* ferrous ammonium sulfate	0.2	g
Sodium chloride	5.0	g
Sodium thiosulfate	0.3	g
Agar	12.0	g
or		
	13.0	g
Phenol red	0.024	g
Distilled water	1.0	liter

Dispense for 2.5-cm butts and 3.8-cm slants. Autoclave at 121°C for 15 minutes.

This medium is recommended for the detection of hydrogen sulfide production by the *Enterobacteriaceae* (see also Peptone iron agar). Inoculate by stabbing into the butt and streaking the slant. Incubate at 37°C and observe daily for 7 days for blackening. Changes in pH in the butt and on the slant are recorded after 18 to 24 hours only.

Trypticase Soy Agar

(Soybean-Casein Digest Agar USP)

Pancreatic digest of casein USP	15	g
Papaic digest of soy meal USP	5	g
Sodium chloride	5	g
Agar	15	g
Distilled water	1	liter

Final pH 7.3

Heat with agitation until the medium boils. Dispense and autoclave at 118° to 121°C for 15 minutes.

Trypticase Soy Agar Slants With Thionine and Basic Fuchsin

Trypticase soy agar is used as a basal medium in which dilutions of thionine and basic fuchsin are incorporated. Thionine and basic fuchsin media prepared as follows:

Prepare 0.1 percent stock solutions of thionine and basic fuchsin in sterile distilled water. These stock solutions may be stored indefinitely under refrigeration. Before adding to the medium, heat each dye solution in a boiling water bath for 20 minutes. While still hot, shake the thionine stock solution and add 5 ml to 1 liter of trypticase soy agar previously sterilized and cooled to approximately 70° to 80°C. Mix the medium well. This results in a final thionine dilution of 1:200,000.

Similarly add 10 ml of basic fuchsin stock solution to another liter of sterilized and cooled trypticase soy agar resulting in a 1:100,000 dilution. Dispense each medium aseptically in 8 ml amounts to standard screw-capped culture tubes. Allow the medium to solidify in a slanted position. Streak duplicate slants of thionine and basic fuchsin with pure growth from a 48 to 72 hour-culture of the *Brucella* sp to be tested. Incubate the slants for 72 hours under increased carbon dioxide tension. Observe for

inhibition of growth by either thionine or basic fuchsin, or by both dyes. Interpret the results by consulting table 9–3.

Trypticase Soy Broth

Pancreatic digest of casein USP	17.0 g
Papaic digest of soy meal USP	3.0 g
Sodium chloride	5.0 g
Dipotassium phosphate	2.5 g
Dextrose	2.5 g
Distilled water	1.0 liter

Final pH 7.3

Dissolve, dispense, and autoclave at 118° to 121°C for 15 minutes.

Tryptone Glucose Extract Agar

Beef extract	3 g
Tryptone	5 g
Glucose	1 g
Agar	15 g
Distilled water	1 liter

Place the above materials in 1 liter of distilled water. Heat to boiling until all ingredients are dissolved. Distribute in suitable containers and sterilize at 15 pounds pressure (121°C) for 15 minutes. Adjust the reaction so that the pH reading after sterilization will be between 6.8 and 7.0.

Tryptophan Broth

Trypticase or tryptone	1 g
Distilled water	100 ml

Mix ingredients thoroughly and sterilize in the autoclave at 121°C for 15 minutes (15 pounds pressure). Store in the refrigerator until ready for use.

Purpose

This is a popular medium used for the detection of indole production. With the addition of 0.01 to 0.1 percent potassium nitrate, the medium may be used to detect nitrate reduction. The indole and nitrate reduction tests are performed on separate cultures in the medium as described in chapter 14.

Urea

Add sufficient distilled water to 31 gm Urea Broth (Difco) to make a total volume of 100 ml. Seitz filter. Add 1.0 ml to 8.0 ml autoclaved fermentation base without the bromthymol blue indicator.

Virulence Agar KL Virulence Agar Base (in Vitro)

Bacto-Proteose peptone	20.0 g
Sodium chloride	2.5 g
Agar	15.0 g
Distilled water	1.0 liter

Final pH 7.8

Suspend 37.5 g of KL virulence agar base in 1,000 ml of distilled water and heat to boiling to dissolve completely. Distribute in 10 ml amounts to standard-sized culture tubes and sterilize in the autoclave for 15 minutes at 15 pounds pressure (121°C). This agar base is used in preparing in vitro virulence agar plates as follows:

Cool the previously autoclaved KL virulence agar tubes to 50° to 55°C in a water bath. Asepically pipette 2 ml of Bacto-KL virulence enrichment and 0.5 ml Bacto-Chapman tellurite solution to a tube containing the 10 ml of KL virulence agar.

Twirl the tube to mix the constituents thoroughly and dispense the medium to a sterile Petri dish. Before the medium solidifies, quickly place a sterile 1 × 7 cm strip of filter paper, previously saturated with diphtheria antitoxin (500 u ml), across the diameter of the plate. This is best accomplished with a pair of sterile forceps or tweezers. Gently probe the strip with the forceps to cause it to settle to the bottom of the melted medium.

Allow the medium to solidify and then preincubate the plate to check for sterility and dry the agar surface. Inoculate the plate with a pure culture suspected of being *Corynebacterium diphtheriae* in the manner described for in vitro virulence testing in chapter 7.

Purpose

The medium is used in testing for toxin production of organisms presumed to be virulent strains of *Corynebacterium diphtheriae*.

Wayne's Phenolphthalein Agar (Sulfatase Test)

1. Dubos oleic agar base (Difco)

Casitone	0.5 g
Asparagine	1.0 g
Disodium phosphate	2.5 g
Monopotassium phosphate	1.0 g
Ferric ammonium citrate	50.0 mg
Magnesium sulfate	10.0 mg
Calcium chloride	0.5 mg
Zinc sulfate	0.1 mg
Copper sulfate	0.1 mg
Agar	15.0 g

Mix ingredients and place 2.3 g in 100 ml of distilled, add 1 ml of glycerol and 65 mg tripotassium salt of phenolphthalein sulfate. Heat this mixture to dissolve and dispense 2-ml portions into small screw-capped vials for 15 minutes. Allow the tubes to harden in an upright position.

2. Sodium carbonate solution:

Sodium carbonate, anhydrous	10.6 g
Distilled water	100.0 ml

Dissolve powder in water until such time as it is used.

Xylose Lysine Desoxycholate (XLD) Agar

Xylose	3.5 g
L-Lysine	5.0 g
Lactose	7.5 g
Sucrose	7.5 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red	0.08 g
Agar (dried)	13.5 g
Sodium desoxycholate	2.5 g
Sodium thiosulfate	6.8 g
Firric ammonium citrate	0.8 g
Distilled water	1.0 liter

Final pH 7.4

Mix the above ingredients and heat with agitation just until the medium boils. Transfer immediately to 45°C to 50°C water bath. Pour into plates as soon as the medium has cooled.

EXPLANATION OF TERMS

Aberrant. Deviating from the normal or typical.

Abscess. A localized collection of pus in a cavity, formed as a result of an infectious process.

Acute Stage. That phase during the course of a disease which is most severe and generally of short duration in patients who recover.

Adherent. Sticking or clinging.

Aerobe. An organism whose growth and reproduction is favored by the *presence* of air or free oxygen.

Aerosol. A suspension of small particles in a gas; for example, the distribution of small particles into the air as a result of a cough or sneeze.

Airborne. Distributed free in the air or atmosphere.

Allergy. Sensitivity to a specific substance which in similar amounts is harmless to most people.

Amorphous. Without visible differentiation in structure.

Amphoteric. Having properties of both an acid and a base.

Anaerobe. An organism whose growth and reproduction are favored by the *absence* of air or free oxygen.

Anaerobic. Having the ability to use oxygen from oxygen compounds for metabolism; living without oxygen.

Anamnestic. Pertaining to previous medical history of a patient.

Anoxia. A condition in which the cells of the body do not have or cannot utilize sufficient oxygen.

Anion. Ion carrying a negative charge.

Anticomplement. A substance combining with, and thus neutralizing, a complement.

Antigen. Any substance which, when introduced into the blood or tissues, incites the formation of antibody and which, when mixed with the antibody, reacts with it is some observable way.

Antiserum. A serum which contains antibody.

Antitoxin. An antibody capable of neutralizing a specific toxin.

Aseptic Technique. Method utilized in an attempt to prevent the introduction or access of microorganisms.

Aspirate. To remove by suction.

Autolysis. Breakdown of all or part of a cell or tissue by self-produced enzymes.

Autolytic. Having the ability to produce autolysis.

Bacteremia. Presence of bacteria in the blood without multiplication or blood destruction.

Bactericidal. Having the capacity to destroy bacteria.

Bacterostatic. Having the capacity to retard the growth of bacteria.

Bacteria. Plural of bacterium.

Bacterium. Any of a group of unicellular, anucleate organisms, placed by *Bergey's Manual of Determinative Bacteriology* in Division II of the Kingdom *Procaryotae*.

Benign. Not malignant.

Biochemical Test. A bacteriological test in which chemicals are employed as indicators of biological activity.

Bipolar. At both poles or ends of the bacterial cell.

Boas Oppler Bacillus. An organism first found in the gastric juice of patients with stomach carcinomas.

Bronchitis. Inflammation of the bronchial tubes.

Bubo. An inflamed swelling of a lymph gland, usually in the armpit or groin.

Butyrous. Growth of butterlike consistency.

Capsule. A gelatinlike envelope surrounding bacteria.

Catheterize. To remove urine from the body of a patient by use of a sterile tube (catheter).

Cation. An ion carrying a positive charge.

Cervicitis. Inflammation of that portion of the female genitourinary tract known as the cervix.

Chain. Four or more bacterial cells attached end to end.

Chancre. A venereal sore or ulcer.

Chemotherapy. The use of chemical agents in the treatment of disease.

Chromogen. Any organic coloring matter.

Chromogenesis. The production of color.

Coagulate. To curdle or clot by chemical action or fermentation.

Complement-Fixation. Complement is inactivated in an antigen-antibody reaction requiring complement for completion.

Concave. Curving in.

Confluent Growth. Bacterial growth in which colonies overlap each other and cannot be successfully picked out individually for subculture.

Conjunctivitis. Inflammation of the inner lining of the membrances which surround the eye.

Contaminant. Any substance or biological agent whose presence results in other sub-

stances or biological agents being impure; that is foreign organisms developing accidentally in a pure culture.

Convalescent Stage. The stage of a disease in which the patient is on the way to recovery.

Convex. Curving out.

Cutaneous. At the surface of the skin.

Cystitis. Inflammation of the bladder.

Debilitating. Producing a weakened condition.

Defibrinate. The removal of fibrin from the blood.

Detergent. A purifying or cleansing agent.

Dextrose. Grape sugar or glucose.

Diplococci. Cocci which occur in pairs.

Disinfectant. An agent used to destroy the vegetative stage of pathogenic microorganisms.

Dissemination. The scattering or distribution, herein applied to microorganisms.

Edema. Swelling, usually resulting from the presence of abnormally large amount of fluid in the intercellular spaces of the body.

Endospores. Thick-walled spores formed within the bacterial cell; for example, typical bacterial spores like those of *B. anthracis* or *B. subtilis*.

Endotoxin. A toxic substance that is retained within the bacterial cell until the cell disintegrates.

Enterotoxin. An exotoxin, excreted by certain bacteria, which is primarily specific in activity toward the intestinal mucosa.

Entire. With an even margin (as applied to colony characteristics).

Enzyme. An organic compound, frequently a protein, capable of influencing a chemical reaction without being structurally changed in the reaction.

Epididymis. The elongated cordlike structure along the posterior border of the testis in the ducts of which the spermatozoa are stored.

Equatorial. Situated about equidistant from each end.

Erythema. Redness of the skin.

Etiologic Agent. The specific causative organism of a disease.

Eucaryote. An organism composed of one or more cells with visible evident nuclei.

Exotoxin. A toxin secreted into the surrounding medium or tissue by intact, living organisms.

Extracellular. Outside of a cell body.

Exudate. A substance which oozes out.

Facultative Aerobe. An organism which prefers to live as an anaerobe but will adapt to aerobic conditions of growth.

Facultative Anaerobe. An organism which prefers to live as an aerobe but will adapt to anaerobic conditions of growth.

False Motility. Moving involuntarily; that is, Brownian movement.

Febrile Stage. That stage of a disease in which the patient manifests elevated temperature.

Fermentation. Decomposition of organic matter by enzymes secreted by microorganisms or other cells.

Fibril. A very small filamentous structure, part of a cell on a fiber.

Filamentous. Growth composed of long, irregularly placed or interwoven threads.

Fix. The process of drying a bacterial slide for staining, using a flame of a burner.

Flagella. Fine hairlike extensions on certain bacteria that are used to propel them thrugh liquids.

Flocculent. Containing small adherent masses of various shapes floating in the culture fluid (usually groups of bacteria).

Fluorescent. Having one color by transmitted light and another by reflected light.

Friable. Easily pulverized or crumbled; as applied to cultures, growth dry and brittle when touched with an inoculating needle.

Genitourinary. Pertaining to the genitals and the urinary organs.

Germicidal. Destroying germs.

Glycopeptides. A carbohydrate having two or more amino acids with a peptide bond linkage.

Granular. Composed of small discrete particles.

Granulomatous. Composed of malignant (cancerous) or nonmalignant growths resembling granulation tissue, a tissue formed in wounds and composed of small, rounded, fleshy masses.

Heat-labile. Destructible by heat.

Heat-stable. Indestructible by heat.

Hemagglutination. Agglutination of erythrocytes; may be caused by an antibody or a viral reaction.

Heme. The deep red iron-containing portion of hemoglobin.

Hemin. A brownish-red crystalline salt of heme formed when hemoglobin is heated with glacial acetic acid and sodium chloride.

Hemodigestive. Pertaining to digestion of the hemoglobin.

Hemolysin. A substance which liberates hemoglobin from red cells.

Hemolysis. Lysis of red blood cells with liberation of hemoglobin. It is indicated by the presence of a zone of hemolyzed red cells around a colony grown on a blood agar. A zone of incomplete (usually green) hemolysis around colonies is termed "alpha" hemolysis. A zone of complete (transparent) hemolysis

around the colonies is termed "beta" hemolysis.

Histochemical. Pertaining to the chemical makeup of cells and tissues.

Homogenize. To subject a specimen to physical processes which result in a mixture of uniform consistency.

Hyaline. Glassy in appearance and transparent or nearly so.

Hyaluronic Acid. An acid mucopolysaccharide found in the ground substance of connective tissue, which acts as a binding and protective agent.

Hydrolysis. A chemical reaction in which a compound reacts with water.

Hypersensitivity. A state of reactivity in which the body reacts to a foreign agent more strongly than is normal.

Inhibitory. Substances which either prevent or greatly reduce the capabilities of a microorganism to grow or reproduce.

Inoculum. A substance introduced by inoculation.

Intraperitoneal. Existing within the peritoneal cavity.

Involution Forms. Retrograde and abnormal forms assumed by microorganisms under certain conditions.

Iridescent. Exhibiting changing rainbow colors in reflected light.

Isoelectric-point (pI). The point at which the electrical potential of a system is balanced.

Isolation. The action of obtaining bacteria in pure culture by subculturing discrete colonies.

Lactose. Milk sugar.

Laryngotracheitis. Inflammation involving both both the larynx and trachea.

L-forms (bacteria). Organisms with deficient cell walls.

Liquefaction. Change of a solid into a liquid.

Lyse. The process of cell destruction which results from the action of specific substances.

Lysin. A specific antibody acting destructively upon cells and tissue.

Lysis. Destruction by a lysin.

Lysogenic. Producing lysins.

Lyophilization. Process of rapidly freezing a substance at an extremely low temperature and then dehydrating in high vacuum.

Macerate. Make soft by steeping in a fluid.

Mastoid. The bony prominence of the temporal bone situated immediately behind the lower half of the external ear.

Mastoiditis. Inflammation within the mastoid cavity.

Membranous. Characterized by a thin and filmlike growth.

Meningitidis. Plural for meningitis.

Meningitis. Inflammation of the membrances covering the brain and or spinal cord.

Meningococcemia. Invasion of the blood stream by meningococci.

Metachromatic. Staining in a different color or shade from what is typical; having the capacity to stain different elements of a cell or tissue in different colors or shades.

Microaerophilic. Preferring low concentrations of oxygen.

Micrometer. A millionth part of a micron; the thousandth part of a millimeter; about 1 25,000 part of an inch.

Morphology. The form and structure, including principally the size and shape of an organism.

Motile. Having the power of motion.

Mucoid. Resembling the gummy, watery liquid that covers mucous membranes.

Mucopurulent. Containing both mucus and pus.

Nasopharynx. That part of the pharynx which lies above the level of the soft palate.

Necrosis. Death of a cell or group of cells which are in contact with living tissue.

Nephritis. Inflammation of the kidney.

Neutralization. The process of checking or counteracting the effects of any agent which produces a morbid effect.

Neurotoxin. A toxin which affects nerve tissue.

Nonchromogenic. Lacking chromogens or coloring matter.

Nonphotochromogenic. Incapable of forming pigment due to light exposure.

Normal Flora. Those living forms which normally occur in a given type of habitat or environment.

Nucleic Acid. Ribonucleic and deoxyribonucleic acids found in the nucleus.

Obligate Aerobe. An organism having the ability to grow only in the presence of air or free oxygen.

Obligate Anaerobe. An organism having the ability to grow only in the absence of air or free axygen.

Opalescent. Showing a milky iridescence.

Opaque. Impervious to light.

Optimum Temperature. The temperature at which grow this most rapid.

Osteomyelitis. Inflammation of bone caused by a microorganism, usually pyogenic.

Otitis Externa. Inflammation of the external ear.

Otitis Interna. Inflammation of the internal ear.

Papule. A small and clearly defined elevated area of the skin.

Parenchymatous. Pertaining to that portion of

an organ which performs its essential functions.

Pathogen. A microorganism capable of causing disease.

Pathogenesis. Origination and development of a disease.

Pellicle. Bacterial growth forming either a continuous or interrupted film at the surface of liquid medium.

Pericardial. Pertaining to the double membranous, fibroserous sac enclosing the heart.

Pericarditis. Inflammation of the membranous sac which contains the heart.

Peritoneum. The glistening and smooth inner lining of the abdominal cavity of the body.

Peritonitis. Inflammation of the peritoneum; a condition usually accompanied by exudation of serum, fibrin, cells, and pus into the peritoneal cavity.

Phagocyte. A cell capable of ingesting bacteria.

Pharynx. The area of the body between the mouth, nares, and esophagus.

Phospholipids. Lipids containing phosphorus which on hydrolysis yield fatty acids, glycerin, and a nitrogenous compound (example: lecithin).

Photogenic. Producing phosphorescence.

Phototrophic. Utilizes light in metabolism, as in certain green plants and bacteria.

Pleomorphic. Different forms within the same species.

Pleuritis. Inflammation of the covering of the lungs with production of fluid in the lung cavity.

Pneumonia (bronchial). Pneumonia characterized by associated inflammation of the bronchial tubes.

Pneumonia (lobar). Pneumonia characterized by inflammation of lung tissue of one or more lobes of the lung.

Pneumonitis. Localized acute inflammation of the lung.

Polar. At the end or pole of the bacterial cell.

Precipitin. An antibody formed in blood serum of an animal due to presence of a soluble antigen usually a protein.

Presumptive Diagnosis. Initial tentative diagnosis of a disease based upon clinical evidence and without other evidence, such as laboratory findings.

Primary Isolation. The initial isolation of microorganisms from a specimen.

Procaryote. A cellular organism that does not have a distinct nucleus.

Proctoscopic Examination. Observation of the lower portion of the intestinal tract by use of an instrument, the proctoscope.

Progeny. Offspring.

Proteolysis. The hydrolysis or decomposition of proteins.

Pseudomembrane. A false membrane; more specifically the membrane produced as a result of activity of the organism which causes diphtheria.

Psychrophiles. Bacteria which thrive best at cold temperatures (between 0°C and 30°C).

Pustule. A small pus-filled elevation at the surface of the skin.

Putrefaction. Decomposition of animal or vegetable proteins by microorganisms.

Pyogenic Organism. An organism characterized principally by the fact that it produces pus.

Pyramidal. Shaped in the form of a pyramid.

Raised. As applied to cultures—growth thick, with abrupt or terraced edges.

Quellung Reaction. A capsular swelling reaction used in the identification of *H. influenzae*, *Klebsiella* sp., *N. meningitidis*, and pneumococci.

Reduction. Removal of oxygen from a compound, addition of hydrogen to a compound, or a gain of electrons or loss in positive valence.

Reniform. In the shape of a kidney.

Reticuloendothelial System. That system of the body which produces blood cells.

Rhizoid. Growth of an irregularly branched or rootlike character, as in *B. mycoides*.

Saprophyte. An organism which normally inhabits certain areas of the body, particularly the gastrointestinal tract, without invading tissue and producing disease.

Satellitism. The tendency to grow as a minute body close to the colony of another organism.

Scotobacteria. Bacteria which has developed an affinity for darkness.

Sensitize. To render more reactive.

Sepsis. Poisoning which is caused by the products of pathogenic microorganism activity.

Septicemia. A morbid condition due to the presence of pathogenic bacteria and their associated toxins in the blood.

Serologic Test. A laboratory test performed on the blood serum of a patient.

Sinusitis. Inflammation within a sinus cavity.

Speculum. An instrument used for the examination of canals and for the collection of vaginal samples.

Subterminal. Situated toward, but not at, the end.

Sucrose. Cane sugar.

Suppurative. Pus-producing.

Synovial. Pertaining to fluid which lubricates the joint.

Systemic. Involving the entire body.

Taxonomy. The orderly classification of organisms into appropriate categories on the basis of relationships among them, with tne application of suitable and correct names.

Tenacious. Holding fast or adhesive.

Terminal. Situated at the extreme end.

Therapeutic Agent. A substance administered in the treatment of a disease.

Toxic. Pertinent to, resembling, or caused by poison.

Toxin. A poisonous substance produced by bacteria (exotoxin, and endotoxin).

Toxoid. A toxin treated so as to destroy its toxicity, but still capable of inducing the formation of antibodies on injection.

Transient. Lasting only a brief period.

Translucent. Semitransparent.

Transparent. As applied to cultures—growth which is water-clear.

True Motility. Moving voluntarily.

Turbid. Cloudy. Turbidity may be uniform, flocculent, or granular.

Ulcer. A loss of substance on a cutaneous or mucous surface, with gradual disintegration and necrosis of the tissues.

Urethritis. Inflammation of the membranous lining of the canal (urethra) which conveys urine from the bladder to the exterior.

Uvula. A small fleshy mass hanging from the soft palate above the root of the tongue.

Vaccination. Inoculation with any vaccine as a preventive measure.

Vaginitis. Inflammation of that portion of the female genital tract known as the vagina.

Viable. Capable of living; specifically, alive and capable of reproducing.

Virulent. Unusually capable of producing disease.

Viscera. Any large internal organ within the three great cavities of the body, especially the abdomen.

Viscous. A thick substance which adheres to the inoculating needle when touched. Sediment which arises as a coherent swirl when liquid media is shaken.

Volutin (granules). Bacterial nucleoprotein occurring as cytoplasmic granules having an affinity for basic dyes.

Zwitterion. An ion that is changed both positively and negatively.

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